The p53 signaling pathway of the large yellow croaker (*Larimichthys crocea*) responds to acute cold stress: Evidence via spatiotemporal expression analysis of *p53*, *p21*, *MDM2*, *IGF-1*, *Gadd45*, *Fas*, and *Akt*

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The *p53* activation is induced by stressors, such as DNA damage, oxidative stress, and activated oncogenes, and can promote cell cycle arrest, cellular senescence, and apoptosis. The large yellow croaker (*Larimichthys crocea*) is an important warm temperate marine fish in the Chinese aquiculture industry. However, few studies have investigated the role of *p53* in the response of *L. crocea* to environmental stressors. Therefore, the aim of the present study was to assess the spatiotemporal mRNA expression levels of genes involved in the *p53* signaling pathway of the large yellow croaker in response to cold stress. The results showed significant changes in the expression levels of *p53*, *p21*, *MDM2*, *IGF-1*, *Gadd45*, *Fas*, and *Akt* in various tissues of the large yellow croaker in response to cold stress for different times. As compared to the control group, *p53* mRNA expression was upregulated in most of the examined tissues at 24 h with the exception of the gill. In the liver, the expression levels of *p53* and *Fas* were significantly decreased at 12 h, while those of *p21*, *MDM2*, *IGF-1*, *Gadd45* were dramatically increased. *Akt* expression was notably changed in response to cold in several tissues. These results suggested that *p53* was potentially a key gene in the large yellow croaker response to cold and possibly other environmental stressors.

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2 **acute cold stress: Evidence via spatiotemporal expression analysis of *p53*, *p21*, *MDM2*, *IGF-***

3 **1*, *Gadd45*, *Fas*, and *Akt***

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15   **Abstract:** The *p53* activation is induced by stressors, such as DNA damage, oxidative stress, and

16   activated oncogenes, and can promote cell cycle arrest, cellular senescence, and apoptosis. The

17   large yellow croaker (*Larimichthys crocea*) is an important warm temperate marine fish in the

18   Chinese aquiculture industry. However, few studies have investigated the role of *p53* in the

19   response of *L. crocea* to environmental stressors. Therefore, the aim of the present study was to

20   assess the spatiotemporal mRNA expression levels of genes involved in the p53 signaling

21   pathway of the large yellow croaker in response to cold stress. The results showed significant

22   changes in the expression levels of *p53*, *p21*, *MDM2*, *IGF-1*, *Gadd45*, *Fas*, and *Akt* in various

23   tissues of the large yellow croaker in response to cold stress for different times. As compared to

24   the control group, *p53* mRNA expression was upregulated in most of the examined tissues at 24

25   h with the exception of the gill. In the liver*,* the expression levels of *p53* and *Fas* were

26   significantly decreased at 12 h, while those of *p21*, *MDM2*, *IGF-1*, *Gadd45* were dramatically

27   increased. *Akt* expression was notably changed in response to cold in several tissues. These

28   results suggested that *p53* was potentially a key gene in the large yellow croaker response to cold

29   and possibly other environmental stressors.

30   **Keywords:** large yellow croaker; cold stress; *p53* signaling pathway; mRNA expression; gene

31   network

1. Introduction

Net cage culturing of the large yellow croaker (*Larimichthys crocea*) is economically important to the marine aquaculture industry in China (Liu and Han, 2011). Over the past decade, low temperature resistance of *L. crocea* has gained considerable attention because of extensive economic losses caused by cold stress in winter, especially in the East China Sea (Xu et al, 2012). In order to breed large yellow croaker with stronger resistance to low temperatures and a lower death rate during the winter season, previous studies have investigated serum expression levels of physiological and biochemical markers, antioxidant production, enzymatic activities, and the proteome in response to cold stress, which showed that the serum biochemical indicators glutamate pyruvate transaminase, glutamic oxaloacetic transaminase, and alkaline phosphatase were significantly affected (Ji et al, 2009; Li et al, 2010; Zhang et al, 2013). More recent studies have focused on genes in response to cold stress. Liver transcriptome analysis indicated that the expression levels of numerous genes were either up- or down-regulated after 12 h of cold stress (Qian and Xue, 2016). Moreover, the expression profile of cold-inducible RNA-binding protein was significantly changed in different tissues of the large yellow croaker during acute cold stress (Miao et al, 2017). As the molecular mechanisms underlying the activation of these genes are complicated, further studies are needed to fully understand the genetic responses of the large yellow croaker to cold stress.

The tumor suppressor *p53* not only plays key roles in the inhibition of cell carcinogenesis and tumor development, but also promotes cell cycle arrest and apoptosis (Levine and Oren, 2009;

53 Kastenhuber and Lowe, 2017), and is involved in autophagy modulation, homeostatic regulation of

54 metabolism, pluripotency, and repression of cellular plasticity (Aylon and Oren, 2016). On account

55 of the negative regulation of *MDM2* (induced by *p53*), basal levels of *p53* are low. The *p53* gene

56 acts as a "guardian of the genome" under physiological conditions (Momand et al, 1992; Haupt et al,

57 1997; Honda et al, 1997; Kubbutat et al, 1997) and is activated by stress signals, such as DNA damage,

58 oncogene activation, and environmental stress. However, the response of *p53* is dependent on the

59 intensity of the stress signal, the cell type, and the stage of cellular differentiation (Horn and

60 Vousden, 2007; Kastenhuber and Lowe, 2017). Notably, the *p53* response is exceedingly flexible, as

61 even a very low basal level of *p53* can protect the cell from the accumulation of DNA damage

62 and subsequent carcinogenesis, which under different stress signals occurs through two typical

63 mechanisms: (1) the promotion of cell senescence and apoptosis in response to severe or constant

64 genotoxic and cellular stressors, and (2) the promotion of temporary cell cycle arrest in order to

65 maintain cell survival prior to DNA repair (Jones et al, 2005; Maddocks et al, 2013; Chen et al, 2013;

66 Pappas et al, 2017).

67   Various target genes of the *p53* signaling pathway involved in the arrest of cellular growth

68 have been investigated, which include growth arrest and DNA damage-inducible protein

69 (*Gadd45*), cyclin-dependent kinase inhibitor 1A (*p21*), and tumor necrosis factor receptor

70 superfamily member 6 (*Fas*) (reviewed in Levine and Oren, 2009). The results of our previous study

71 showed that the *p53* signaling pathway was significantly enriched in the liver of the large yellow

72 croaker in response to cold stress for 12 h, while numerous genes related to cell cycle arrest,

73 apoptosis, and DNA repair and damage prevention were remarkably affected (Qian and Xue, 2016).

74 Reportedly, *p53* promotes apoptosis in the gills of the Nile tilapia (*Oreochromis niloticus*) and

75 zebrafish (*Danio rerio*) in response to cold stress (Wang, 2016). Similarly, *p53* mRNA expression

76 was significantly upregulated in the muscle tissue of *D. rerio* under low temperature stress (Li et

77 al, 2018).

78 Therefore, the aim of the present study was to investigate the role of *p53* in response to cold-

79 induced stress via analysis of the spatiotemporal expression of various genes (i.e., *p53*, *p21*,

80 *MDM2*, *IGF-1*, *Gadd45*, *Fas*, and *Akt*) in different tissues of the large yellow croaker.

81 **2. Materials and methods**

82 *2.1 Ethics statement*

83 The study protocol was approved by the Medical Ethics Committee of Taizhou University

84 Medical College (TZYXY2019-211) and conducted in accordance with the guidelines of the

85 Institutional Animal Care and Use Committee. To minimize suffering, all experiment fish in this

86 study were anesthetized by Tricaine-S (TMS, MS-222)(50 mg /L) firstly. And then we collected

87 the tissues of large yellow croaker after the fish lost consciousness. Lastly, the fish which has

88 been taken a surgery would be sprayed with anesthetic (200 mg/L), and let it death with

89 euthanasia.

90 *2.2 Fish and induction of acute cold stress*

91   A total of 180 large yellow croakers (mean weight, 80 ± 0.7 g) were purchased from a

92   mariculture farm located in Xiangshan Bay (Zhejiang Province, China), randomly assigned to

93   one of six groups (30 fish/group), and then cultured in 500-L plastic aerated tanks in the

94   laboratory of the Ningbo Marine and Fishery Science and Technology Innovation Base (Zhejiang

95   Province, China) for 7 days. During the adaption period, the fish were fed granulated feed twice

96   per day (04:30 and 18:30 h). A total of 90 fish cultured in three tanks were exposed to acute cold

97   stress with the use of ice wrapped in thick plastic bags until the water temperature decreased to

98   14°C within 2 h (cold stress group), while the other 90 fish in three tanks were cultured at

99   environmental temperature and received no treatment (control group). After 1, 3, 6, 12, 24, 48,

100 and 72 h of acute cold stress, the liver, muscle, gill, heart, spleen, intestine, brain, and kidney

101 were collected from three fish in the cold stress group and control group, respectively, and

102 immediately snap-frozen in liquid nitrogen, then stored at -80°C.

103 *2.3 Total RNA extraction and cDNA synthesis*

104 Total RNA was extracted from each sample using the E.Z.N.A.® Total RNA Kit I (Omega Bio-

105 Tek, Inc., Norcross, GA, USA) in accordance with the manufacturer's instructions. Total RNA

106 was quantified with a NanoDrop™ 1000 Spectrophotometer (NanoDrop Technologies, LLC,

107 Wilmington, DE, USA) and RNA integrity was assessed with the use of an Agilent 2100

108 Bioanalyzer (Agilent Technologies, Inc., Santa Clara, CA, USA). The RNA integrity value of all

109 samples was ≥ 8. The extracted RNA was stored at -80°C until analysis.

110   First stand cDNA was synthesized from total RNA using the PrimeScript™ RT reagent Kit

111   with gDNA Eraser (Takara Bio, Inc., Kusatsu, Shiga Prefecture, Japan) in accordance with the

112   manufacturer's instructions and stored at -20°C until quantitative real-time polymerase chain

113   reaction (qRT-PCR) analysis.

114   *2.4 Spatiotemporal expression analysis*

115   The qRT-PCR analyses of the spatiotemporal expression profiles of *p53*, *p21*, *MDM2*, *IGF-1*,

116   *Gadd45*, *Fas*, and *Akt* were conducted with primers designed using Primer Premier 5 software

117   (Premier Biosoft, Palo Alto, CA, USA) (Table 1). β-actin was used as a housekeeping gene.

118   Before qRT-PCR, the amplification efficiency of the primers was evaluated with five 10-fold

119   serial dilutions of cDNA of all tissues.

120   The qRT-PCR analysis was performed using a CFX96 Real-Time PCR System (Bio-Rad

121   Laboratories, Hercules, CA, USA) with a total reaction volume of 20 μL, consisting of 1 μL of

122   cDNA diluted to 1:5 with sterile DNase/ RNase-free distilled water, 0.6 μL of the forward primer,

123   0.6 μL of the reverse primer, 9 μL of FastStart Universal SYBR Green Master mix (Sigma-

124   Aldrich Corporation, St. Louis, MO, USA), and 8.8 μL of sterile DNase/RNase-free distilled

125   water. The following thermal cycling conditions were used: 95°C for 10 min followed by 40

126   cycles at 95°C for 15 s, 58°C for 20 s, and 72°C for 20 s. A melting curve was generated. Each

127   sample was amplified in triplicate and the relative expression levels of *p53*, *p21*, *MDM2*, *IGF-1*,

128   *Gadd45*, *Fas*, and *Akt* were normalized to that of *β-actin* with the $2^{-\Delta\Delta CT}$ method (Thomas and

129   Livak, 2008). Statistical significance was determined using one-way analysis of variance. All

130   statistical analyses were performed using IBM SPSS Statistics for Windows, version 21.0. (IBM

131   Corporation, Armonk, NY, USA). A probability (p) value of < 0.05 was considered statistically

132   significant and < 0.01 as highly significant. All qPCR data could be obtained in "Supplemental

133   File" which named with raw data.

134   **3. Results**

135   *3.1 Analysis of the p53 signaling pathway*

136   The results of our previous study showed that the expression profiles of genes involved in the

137   *p53* signaling pathway were significantly affected and this pathway was remarkably enriched in

138   the liver transcriptome of the large yellow croaker in response to acute cold stress for 12 h (Qian

139   and Xue, 2016). Gene networks associated with cell cycle arrest, apoptosis, *p53* negative feedback,

140   and DNA repair and damage prevention were evaluated in the present study based on the

141   transcriptome data of previous studies (Fig. 1).

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143   *3.2 The mRNA profiles of Akt, MDM2, p53, p21, Gadd45, Fas, and IGF-1 in liver tissue*

144   The mRNA expression profiles of genes and gene networks in the liver of the large yellow

145   croaker in response to acute cold stress were investigated (Fig. $2a_{1-7}$, b). Liver mRNA expression

146   levels of *p53* were significantly decreased in response to cold stress at 3, 6 and 72 h, while

147   significantly increased at 24 and 48 h. In addition, the mRNA levels of *Akt*, *MDM2*, *p21*, and

148   *Gadd45* were significantly increased at 1 and 3 h, while the expression levels of *p21* and *Gadd45*

149   were significantly upregulated with the exception of 48 h. There was a good agreement between

150   the qRT-PCR findings and previous liver transcriptome data of the large yellow croaker in

151   response to acute cold stress at 12 h. The changes in mRNA expression levels of the selected

152   genes were comparable between the two methods, although *IGF-1* expression was increased by

153   qRT-PCR, while decreased according to the transcriptome data. The qRT-PCR results showed

154   that the mRNA expression levels of *Akt*, *MDM2*, *p21*, and *Gadd45* were significantly increased,

155   while that of *p53* was increased after 12 h of acute cold stress, as compared to the control group,

156   although this difference was not statistically significant.

157   *3.3 Spatiotemporal expression patterns of Akt, MDM2, p53, p21, Gadd45, Fas, and IGF-1*

158   The spatiotemporal expression profiles of *Akt*, *MDM2*, *p53*, *p21*, *Gadd45*, *Fas*, and *IGF-1* in

159   sampled tissues of large yellow croaker were determined by qRT-PCR analysis. The results of

160   qRT-PCR analysis are shown in Figs. $2a_{1-7}$ and 2b (liver), $3a_{1-7}$ and 3b (muscle), $4a_{1-7}$ and 4b

161   (brain), $5a_{1-7}$ and 5b (spleen), $6a_{1-7}$ and 6b (gill), $7a_{1-7}$ and 7b (kidney), $8a_{1-7}$ and 8b (intestine),

162   and $9a_{1-7}$ and 9b (heart). *p53* mRNA expression levels in muscle were obviously increased after 1,

163   6, 12, 24, and 72 h of acute cold stress, but were significantly decreased at 3 h. mRNA levels of

164   *Gadd45* and *p21* were increased in muscle throughout the cold stress period, although *Gadd45*

165   expression was downregulated at 3 h. The mRNA expression levels of *Akt* and *Fas* were

166 downregulated, while those of *MDM2*, *p53*, *p21*, *Gadd45*, and *IGF-1* were upregulated in muscle

167 tissue at 12 h (Fig. 3a$_{1-7}$, b).

168   Brain mRNA expression levels of all selected genes were significantly affected after 1 and 48

169 h of acute cold treatment. Notably, *p53* expression was significantly increased at 1, 12, 24, and

170 48 h, but not at 3, 6, and 72 h. Also, the expression levels of *MDM2* and *Gadd45* were

171 dramatically upregulated in the brain tissue of the large yellow croaker throughout most of the

172 treatment period (Fig. 4a$_{1-7}$, b).

173   In contrast to the brain tissue, *p53* mRNA expression in the spleen tissue was obviously

174 increased at 24 and 48 h, but not at all times. Spleen mRNA expression levels of *Gadd45* were

175 increased after cold stress, although there was no statistical significance at 72 h. In addition, the

176 expression levels of *Akt*, *MDM2*, *p53*, *p21*, *Gadd45*, and *IGF-1* were significantly upregulated at

177 24 h, while that of *Fas* was significantly downregulated (Fig. 5a$_{1-7}$, b).

178   In the gill tissues, *p53* expression was significantly downregulated at 1, 3, 48, and 72 h, and

179 significantly upregulated at 6 and 12 h. *MDM2* and *Akt* mRNA expression levels were obviously

180 increased at 1, 24, 48, and 72 h, and at 3, 6, and 12 h of cold stress, respectively. *MDM2* mRNA

181 expression was not significantly affected, while *Akt* expression was significantly decreased. Gill

182 mRNA expression levels of *p21* were significantly increased throughout the cold stress period, as

183 was that of *Gadd45* with the exception at 72 h (Fig. 6a$_{1-7}$, b).

184   Kidney mRNA expression levels of *p21* and *Gadd45* were significantly increased throughout

185   the acute cold stress period, while that of *Fas* was obviously decreased with the exception of 1

186   and 24 h. The mRNA expression levels of *p53* in the kidney tissues were significantly

187   upregulated at 1, 6, 12, 24, and 48 h. *MDM2* mRNA expression was upregulated throughout the

188   cold stress period, although there was no significant change at 6 and 12 h (Fig. 7a$_{1-7}$, b).

189   The mRNA expression of *p53* in the intestinal tissue was significantly upregulated at 1, 24,

190   and 48 h, but downregulated at 3, 6, 12, and 72 h. At 24 and 48 h after acute cold stress, the

191   mRNA expression levels of *Akt*, *MDM2*, *p53*, *p21*, *Gadd45*, *Fas*, and *IGF-1* were all

192   significantly increased. At 72 h, the mRNA expression levels of *Akt*, *MDM2*, *p21*, *Gadd45*, and

193   *IGF-1* were significantly upregulated, while those of *p53* and *Fas* were downregulated (Fig. 8a$_{1-7}$,

194   b).

195   The qRT-PCR results indicated that in the heart tissues, the mRNA expression levels of *Akt,*

196   *MDM2*, *p53*, and *Gadd45* were significantly increased, while *p53* was significantly down-

197   regulated at 3 h with no significant difference at 1 and 12 h (Fig. 9a$_{1-7}$, b).

198   **4. Discussion**

199   Low temperatures that exceed the tolerance range of fish are known to disrupt energy

200   metabolism, biochemical composition, immune function, and gene expression (Lu et al, 2019; Song

201   et al, 2019). However, few studies have investigated the specific molecular mechanisms

202   underlying the physiological changes in large yellow croaker in response to acute cold stress.

203   The results of our previous study of the liver transcriptome of *L. crocea* in response to 12 h of

204   acute cold stress, identified a large number of differentially expressed genes that were enriched

205   in the *p53* signaling pathway. Specifically, the mRNA expression levels of *MDM2*, *p21*, *Gadd45*,

206   *CytC,* and *Apaf-1* were significantly increased, while those of *Fas*, *IGF*, and *CDK4/6* were

207   significantly decreased. In addition, there was no significant change in the mRNA expression

208   levels *p53* or other genes related to cell cycle arrest, apoptosis, inhibition of angiogenesis and

209   metastasis, DNA repair and damage prevention, and *p53* negative feedback (Qian and Xue, 2016).

210   In the present study, acute cold stress altered the expression profiles of genes related to cell cycle

211   arrest and apoptosis in the liver, muscle, brain, spleen, gill, kidney, intestine, and heart. Of

212   interest, the expression levels of *p21* and *Gadd45*, which are related to cell cycle arrest, were

213   significantly changed in the liver, muscle, and kidney tissues throughout the cold stress period,

214   while those of *Fas* and *IGF-1*, which are related to apoptosis, were also significantly altered in

215   the heart tissue.

216   As a guardian of the genome, *p53* is remarkably sensitive to environmental factors and is

217   readily activated by multiple stress signals, especially in aquatic organisms in response to

218   temperature change. Li et al. (2018) demonstrated that upregulation of *p53* expression in response

219   to low temperature stress can cause tail malformation of the zebrafish. The molecular mechanism

220   of the *p53* pathway in response to cold stress also involves *MDM2* (Wang, 2016; Sun et al, 2019). In

221   this study, the mRNA expression levels of *p53* and *MDM2* were significantly increased in the

222   brain tissue of the large yellow croaker after 1 h of acute cold stress. Although there was no

223   change in the *p53* expression profile, *MDM2* expression was increased at 3 and 6 h. *p53* induces

224   the expression of *MDM2* and *MDM2* inhibits the activity and promotes the degradation of *p53* in

225   a negative feedback loop (Barak et al, 1993; Wu et al, 1993, Picksley and Lane, 1993). In the present

226   study, the expression levels of *p53* and *MDM2* in response to the same cold stress period differed

227   among tissues. In contrast to that observed in the brain, muscle, kidney, and intestine, there was

228   no significant change in *p53* mRNA expression in the liver after 1 h of cold stress, while *MDM2*

229   mRNA expression was significantly upregulated in the liver, heart, and spleen. There results may

230   be due to the increased expression levels of *p53* in the liver (1.29-fold), heart (1.06-fold), and

231   spleen (1.77-fold), which may have also impacted the expression of *MDM2* after 1 h of cold

232   stress. In addition, the upregulation of *MDM2* inhibited *p53* expression in the liver at 3 and 6 h.

233   *Akt* plays key roles in glucose metabolism, apoptosis, cell proliferation, transcription, and cell

234   migration. Activated *Akt* inhibits apoptosis through *MDM2* phosphorylation, which then

235   inactivates *p53* (Song et al, 2005; Farrell et al, 2009). It has been reported that decreased mRNA

236   expression of *Akt* and increased expression of *p53* can cause apoptosis of hepatocytes, suggesting

237   an inverse correlation between these two genes (Wu et al, 2016). A delicate relationship between

238   *Akt* and *p53* also occurred in the liver of the large yellow croaker after 1, 3, and 12 h of cold

239   stress. During the cold stress period, the expression levels of *Akt* and *MDM2* were significantly

240   increased, whereas *p53* expression was unchanged or decreased (Fig. 2b). In the present study,

241   the relationships among *Akt*, *MDM2*, and *p53* seemed to be more intricate in different tissues in

242   response to acute cold stress. *Akt* mRNA expression in the brain was significantly increased at 1

243   h, returned to normal levels from 3 to 6 h, decreased at 12 h, and increased again from 24 to 72 h,

244   while *MDM2* expression was upregulated during most of the cold stress period, but not at 12 h

245   (Fig. 4b). These results indicated that *MDM2* was regulated by *Akt* as well as *p53*.

246   *Fas* and *p21* are target genes of the *p53* pathway. The activation of p21 usually predicts the

247   beginning of cell cycle arrest, whereas *Fas* promotes apoptosis. Previous studies have reported

248   that short pulses of *p53* activity usually lead to cell cycle arrest, as the p21 promoter is more

249   sensitive to this signaling output, while sustained *p53* signaling usually leads to changes in *Fas*

250   expression, resulting in apoptosis. However, the mRNA signal of *p53*, either short or sustained,

251   had no impact on the maximal level of the translated *p53* protein (Espinosa et al, 2003; Gomes et al,

252   2010; Morachis et al, 2010; Kastenhuber and Lowe, 2017). In this study, hepatic mRNA levels of *p53*

253   increased by 1.29-fold in response to cold stress at 1 h. Although this change was not statistically

254   significant, this slight increase in *p53* expression could be sufficient to result in a change in *p21*

255   expression. In addition, mRNA expression of *p21* was remarkably increased from 1 to 24 h, even

256   at 72 h, although *p53* expression was decreased, while *Fas* expression was decreased or

257   remained comparatively unchanged. It is not clear why there were such differences in the

258   expression patterns of *p21* and *Fas*, as it seems that targeting of *p53* by these genes had no

259   impact on the expression profiles of *p53* at 3, 6, 12, 48, and 72 h in the liver tissues. Similar

260   changes in expression levels occurred in the other tissues at certain times. One possibility could

261   be complex regulatory signals from other genes that may be unknown in the *p53* signaling

262   pathway in response to acute cold stress, which could affect the expression levels of *p21* and *Fas*.

263   Hence, further studies are warranted to fully understand the molecular mechanism of all of genes

264   involved in the *p53* signaling pathway.

265   *Gadd45* is as an important carcinogenic stress response factor that is sensitive to physiological

266   and environmental stressors, and usually induced by cell cycle stagnation, DNA damage, and

267   apoptosis (Liebermann and Hoffman, 2008; Salvador et al, 2013; Peng et al, 2015). It has been observed

268   that *Gadd45* can interact with *p21*, which activates *p53* via *p38* to maintain *p53* signaling (Smish

269   et al, 1994; Vairapandi et al, 1996; Azam et al, 2001; Liebermann and Hoffman, 2008). In the present study,

270   *Gadd45* was sensitive to both long- and short-term cold stress, as indicated by the consistently

271   high expression levels in the liver, intestine, kidney, and muscle at all time points. Even in the

272   other tissues (spleen, heart, and gill), *Gadd45* mRNA expression was significantly increased at

273   most time points. *Gadd45* is known to prevent DNA damage and promote DNA repair (Peng et al,

274   2015). It was possible that DNA damage was induced by acute cold stress, which resulted in

275   significant increases in *Gadd45* expression. In addition, increased *Gadd45* expression may

276   impact *p21* expression as mentioned previously.

277   The results of the present study indicated that genes involved in the *p53* signaling pathway

278   were largely affected by acute cold stress. There were significant changes in the mRNA

279   expression levels of *Akt*, *MDM2*, *p53*, *p21*, *Gadd45*, *Fas*, and *IGF-1* in the liver, brain, muscle,

280   gill, kidney, intestine, heart, and spleen in response to acute cold stress. *p53* target *p21* and

281   *Gadd45*, which are involved with cell cycle arrest and were more sensitive to cold stress than

282   *Fas*. mRNA expression of *Gadd45*, which is involved in DNA repair, was significantly increased

283   in most of the studied tissues (liver, muscle, kidney, and intestine) in response to cold stress. The

284   results of this study are in agreement with those of prior studies, which reported that genes

285   involved in the *p53* signaling pathway could be affected by acute cold stress. However, further

286   studies are needed to elucidate the molecular mechanisms of genes in the *p53* signaling pathway

287   that are activated by low temperature stress.

288   **Acknowledgements**

292   **Conflict of interest**

293   We declare that we have no conflicts of interest.

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377   **Legends:**

378   **Fig. 1.** Putative gene networks in large yellow croaker stressed by 12 h acute cold based on the transcriptome
379   data of previous studies. Enriched gene networks associated with cell cycle arrest, apoptosis, p53 negative
380   feedback, and DNA repair and damage prevention. Red indicates up-regulated, blue down-regulated, white
381   indicates no changes. Full names of abbreviated genes are listed in Abbreviation.
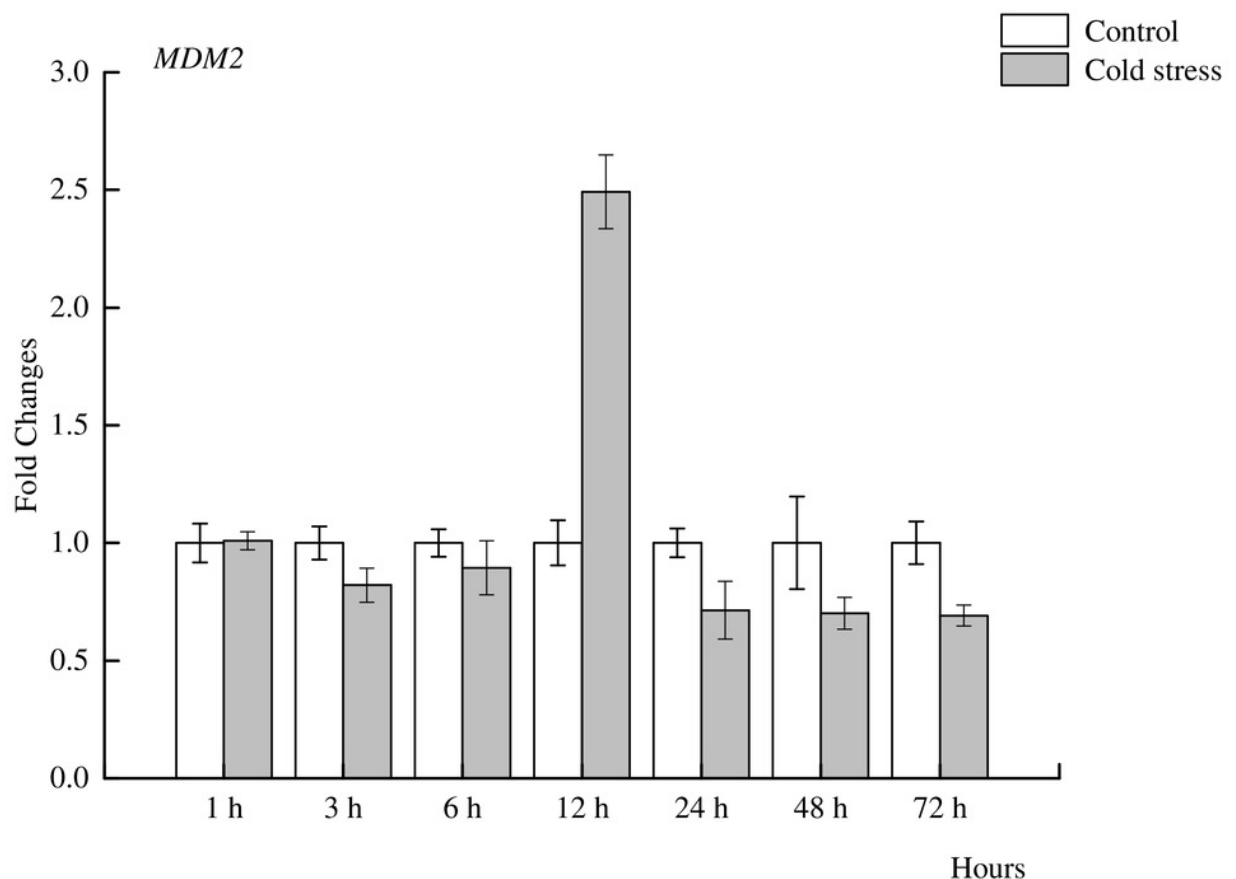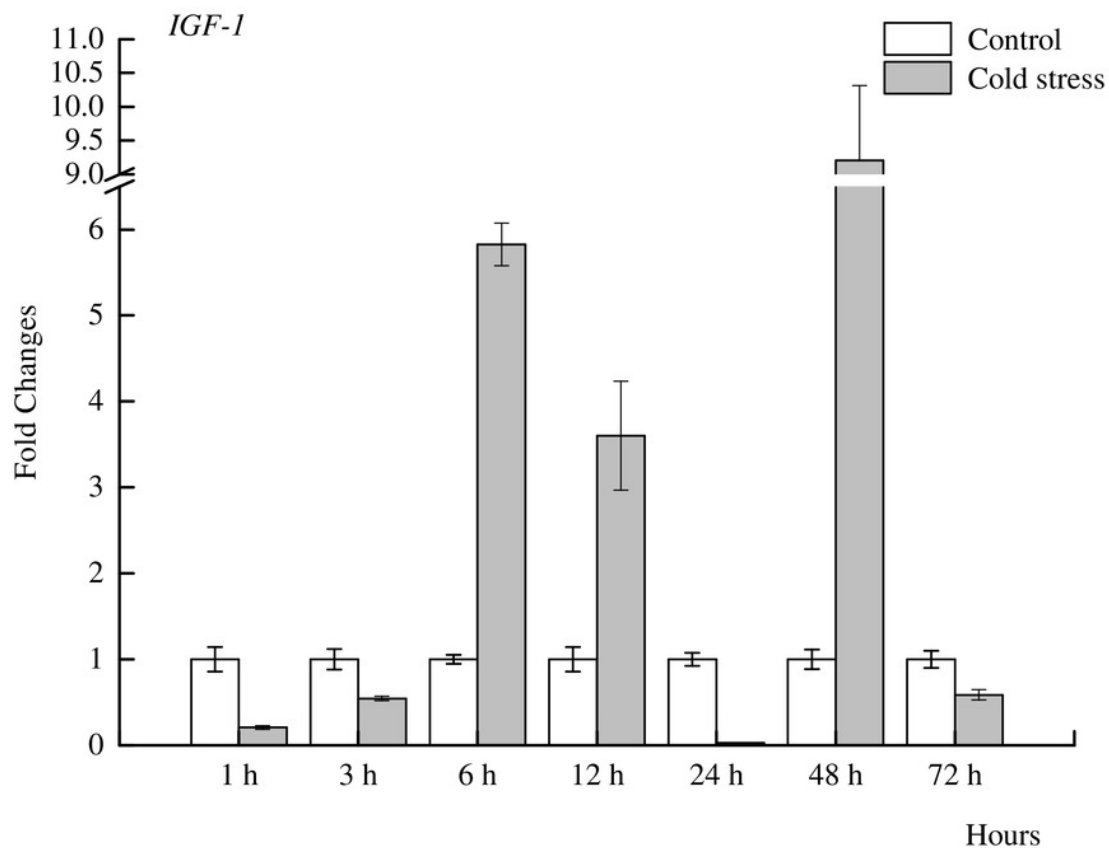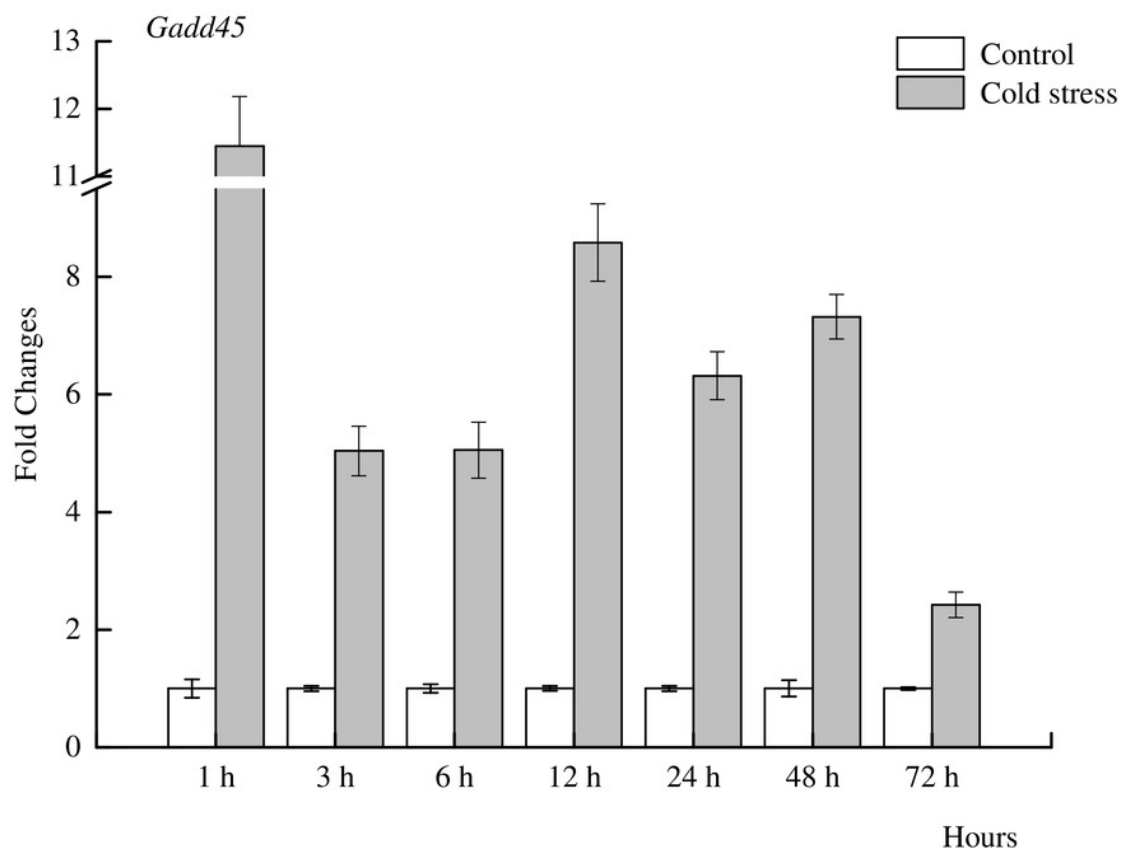382
383   **Fig. 2.** qPCR analysis of genes in the liver of large yellow croaker under acute cold stress at different time
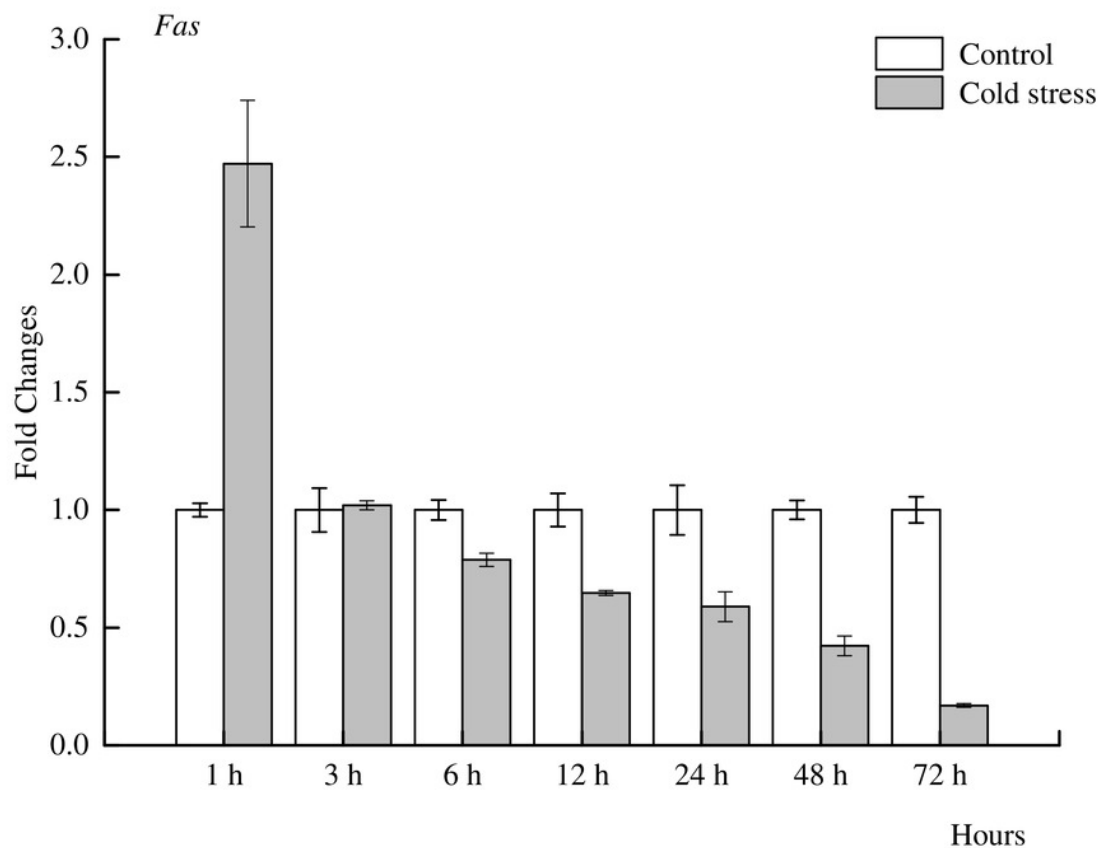384   point (1, 3, 6, 12, 24, 72 h) (Fig. 2a$_{1-7}$); putative gene networks based on qPCR datas (Fig. 2b). Namely, tumor
385   protein p53 (*p53*), protein kinase B (*Akt*), insulin-like growth factor 1 (*IGF-1*), tumor necrosis factor receptor
386   superfamily member 6 (*Fas*), cyclin-dependent kinase inhibitor 1A (*p21*), growth arrest and DNA damage-
387   inducible protein (*Gadd45*), E3 ubiquitin-protein ligase mdm2 (*MDM2*).The results are expressed as mean fold
388   change ± SD (n = 3 fish per treatment). Significant differences were considered at *$0.01 < P \leq 0.01$ and **$P <$
389   0.01.
390
391   **Fig. 3.** qPCR analysis of genes in the muscle of large yellow croaker under acute cold stress at different time
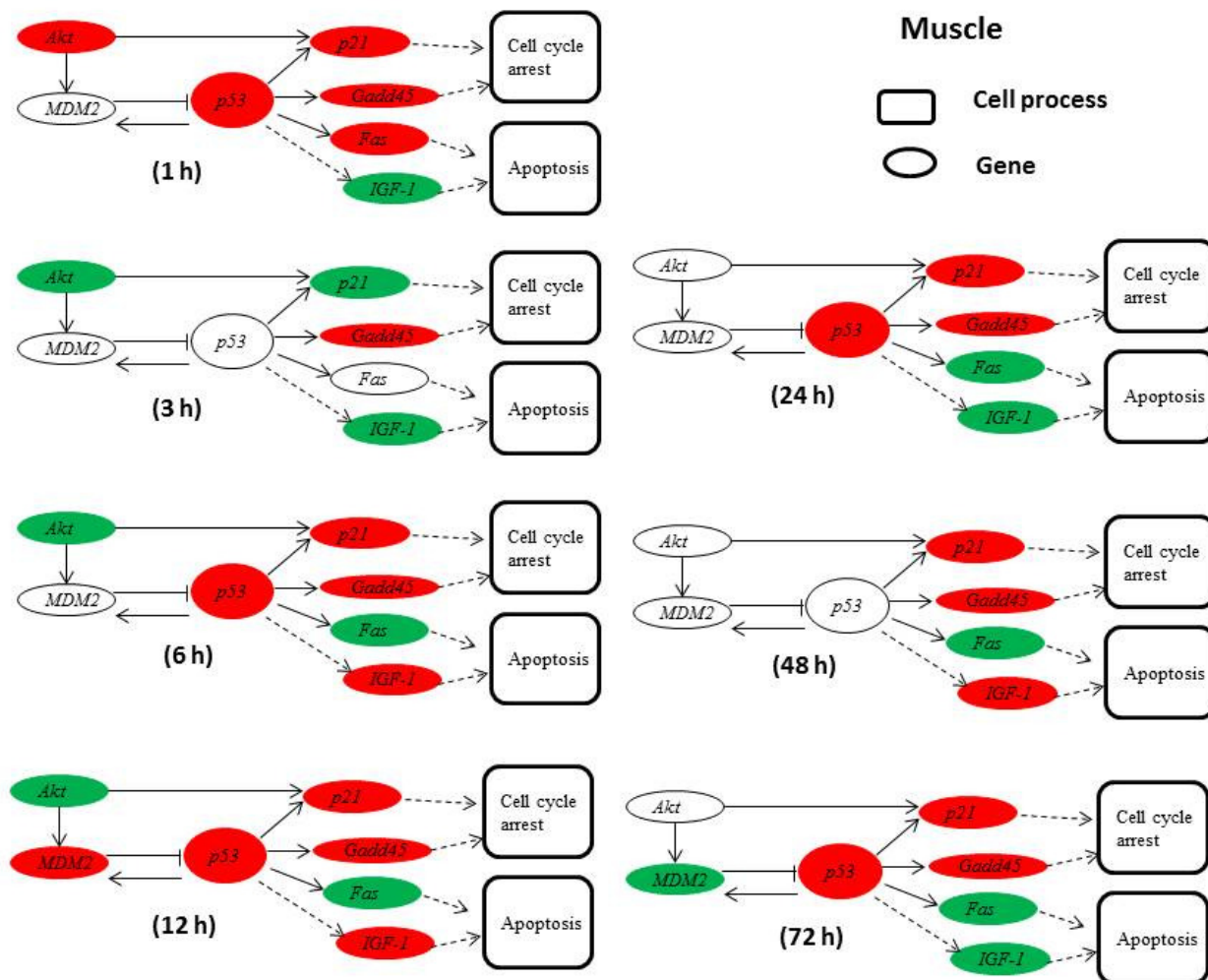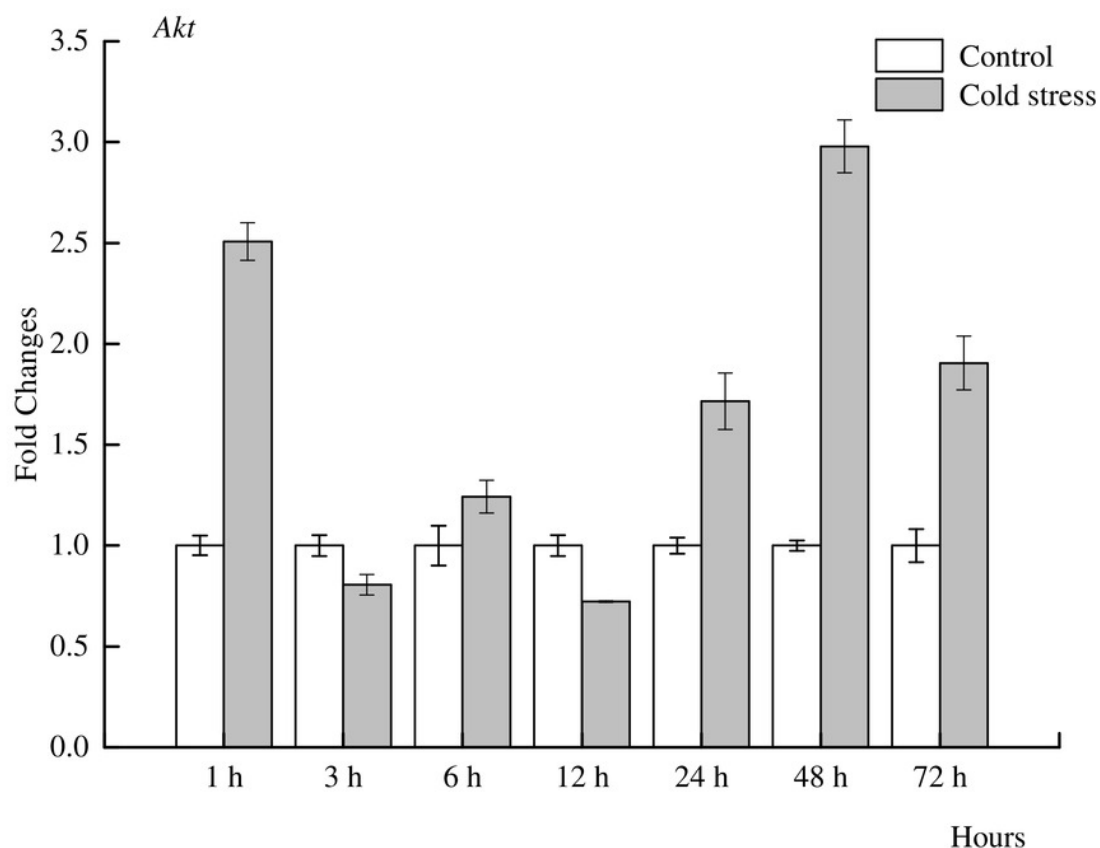392   point (1, 3, 6, 12, 24, 72 h) (Fig. 3a$_{1-7}$); putative gene networks based on qPCR datas (Fig. 3b).
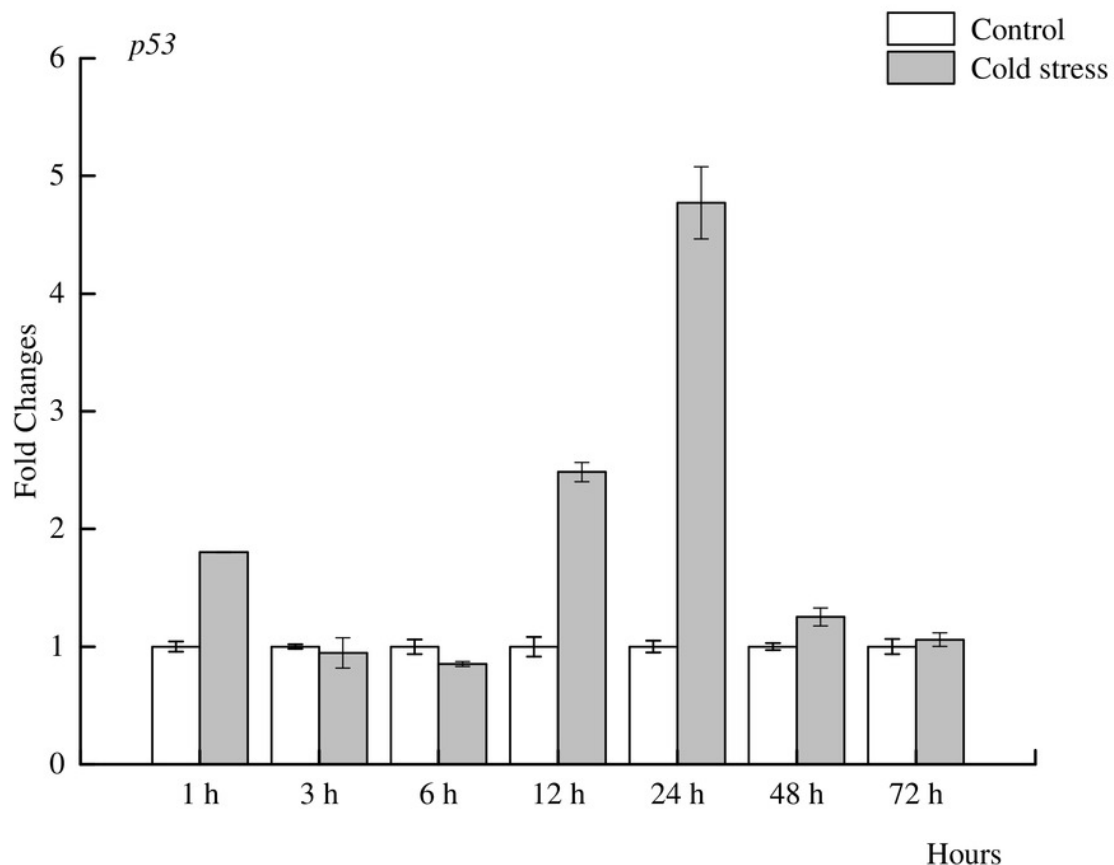393
394   **Fig. 4.** qPCR analysis of genes in the brain of large yellow croaker under acute cold stress at different time
395   point (1, 3, 6, 12, 24, 72 h) (Fig. 4a$_{1-7}$); putative gene networks based on qPCR datas (Fig. 4b).
396
397   **Fig. 5.** qPCR analysis of genes in the spleen of large yellow croaker under acute cold stress at different time
398   point (1, 3, 6, 12, 24, 72 h) (Fig. 5a$_{1-7}$); putative gene networks based on qPCR datas (Fig. 5b).
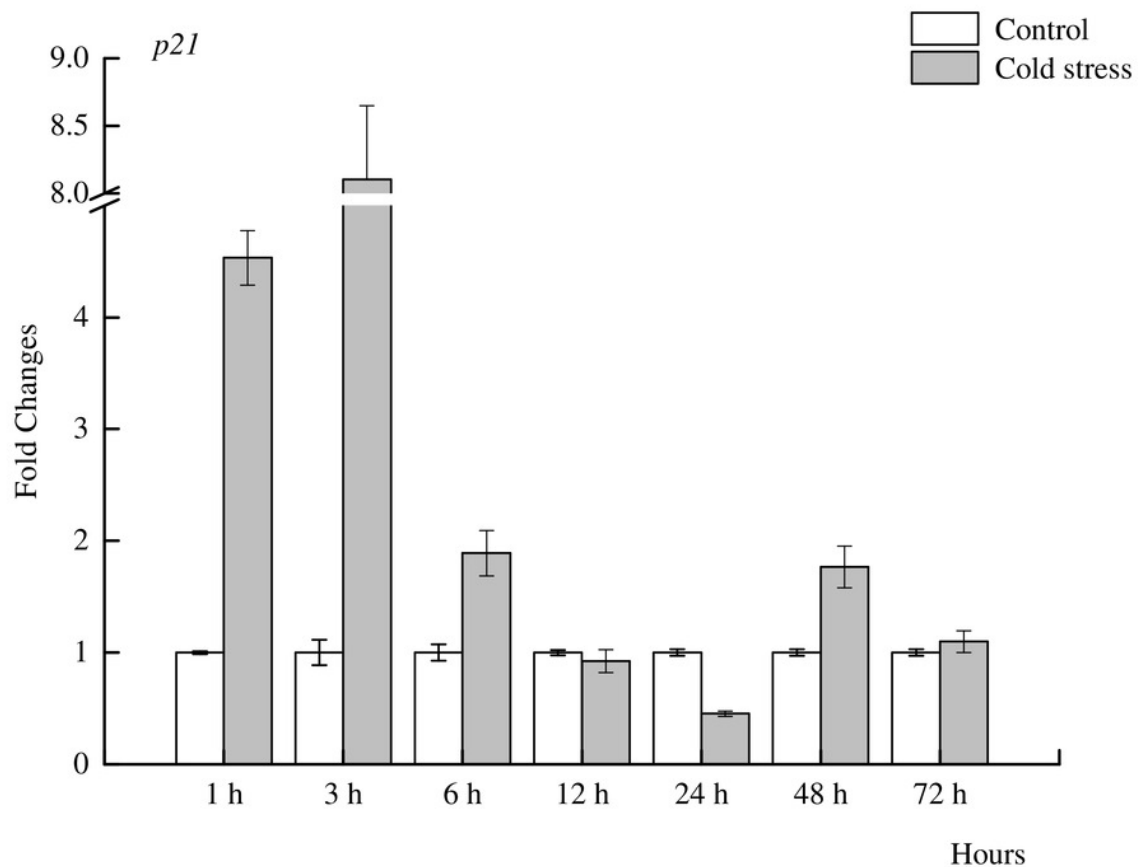399
400   **Fig. 6.** qPCR analysis of genes in the gill of large yellow croaker under acute cold stress at different time point
401   (1, 3, 6, 12, 24, 72 h) (Fig. 6a$_{1-7}$); putative gene networks based on qPCR datas (Fig. 6b).
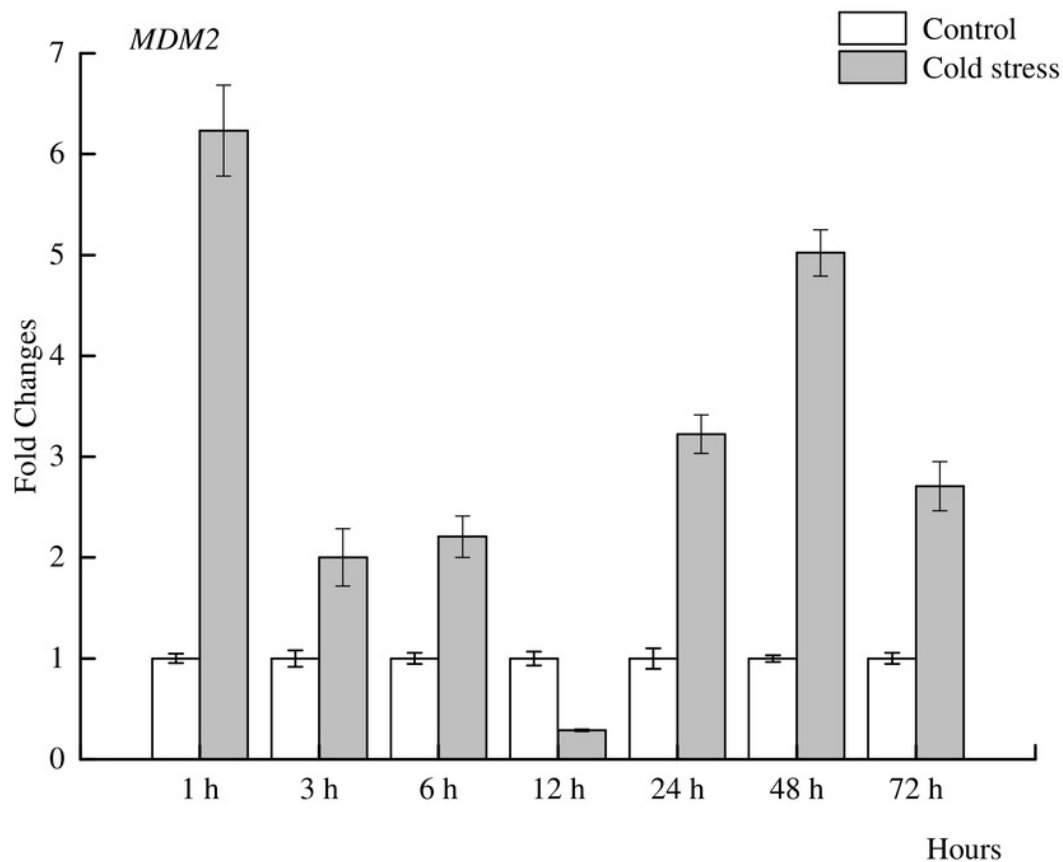402
403   **Fig. 7.** qPCR analysis of genes in the kidney of large yellow croaker under acute cold stress at different time
404   point (1, 3, 6, 12, 24, 72 h) (Fig. 7a$_{1-7}$); putative gene networks based on qPCR datas (Fig. 7b).
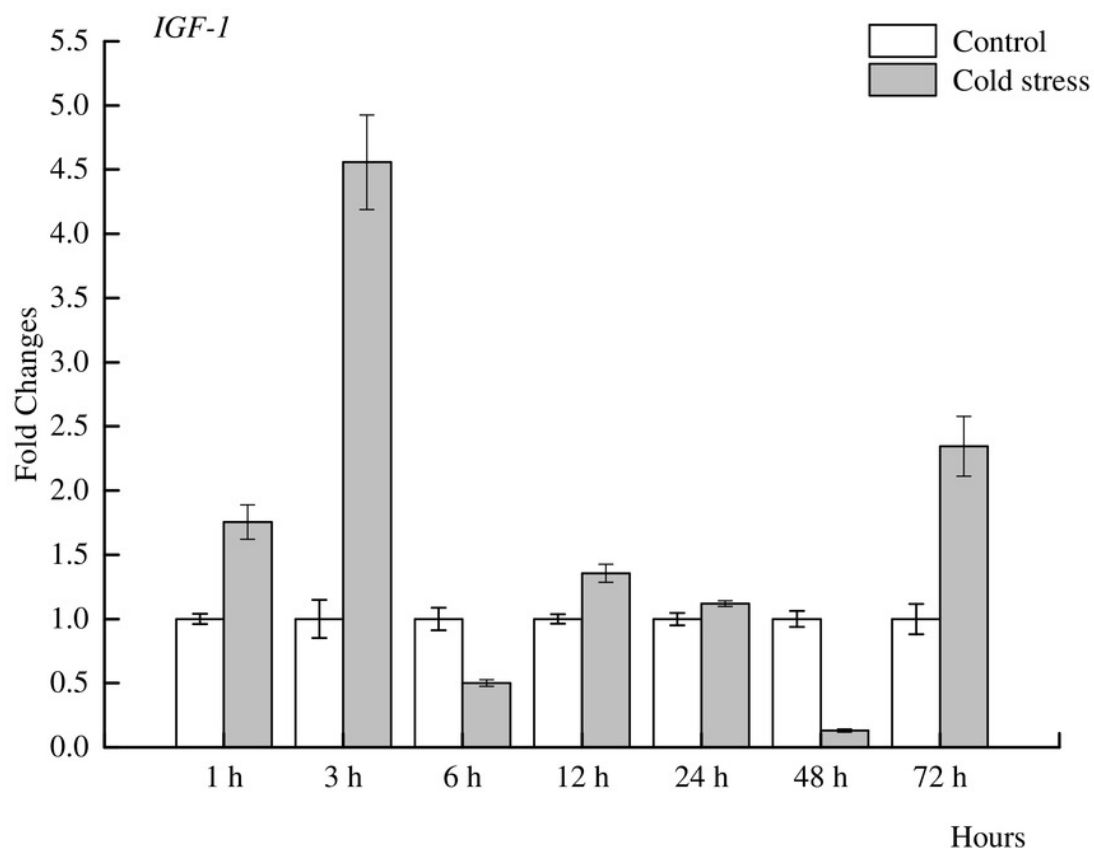405
406   **Fig. 8.** qPCR analysis of genes in the intestine of large yellow croaker under acute cold stress at different time
407   point (1, 3, 6, 12, 24, 72 h) (Fig. 8a$_{1-7}$); putative gene networks based on qPCR datas (Fig. 8b).
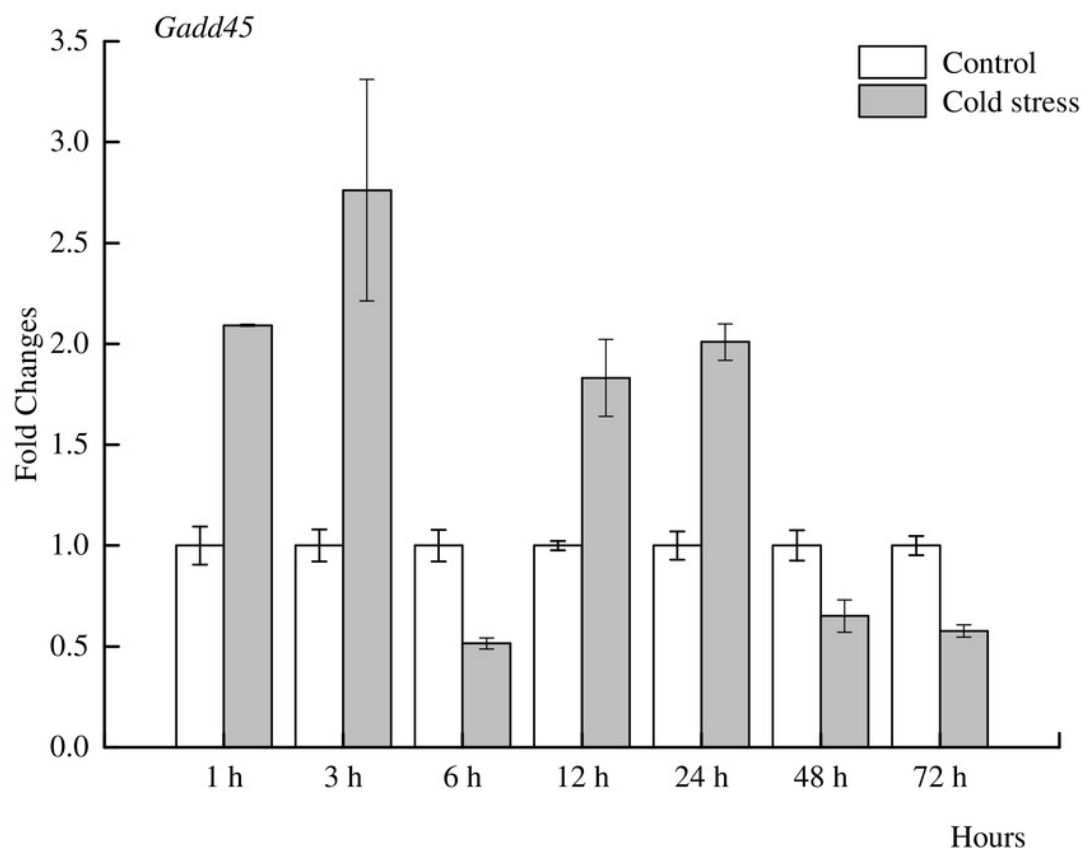408
409   **Fig. 9.** qPCR analysis of genes in the heart of large yellow croaker under acute cold stress at different time
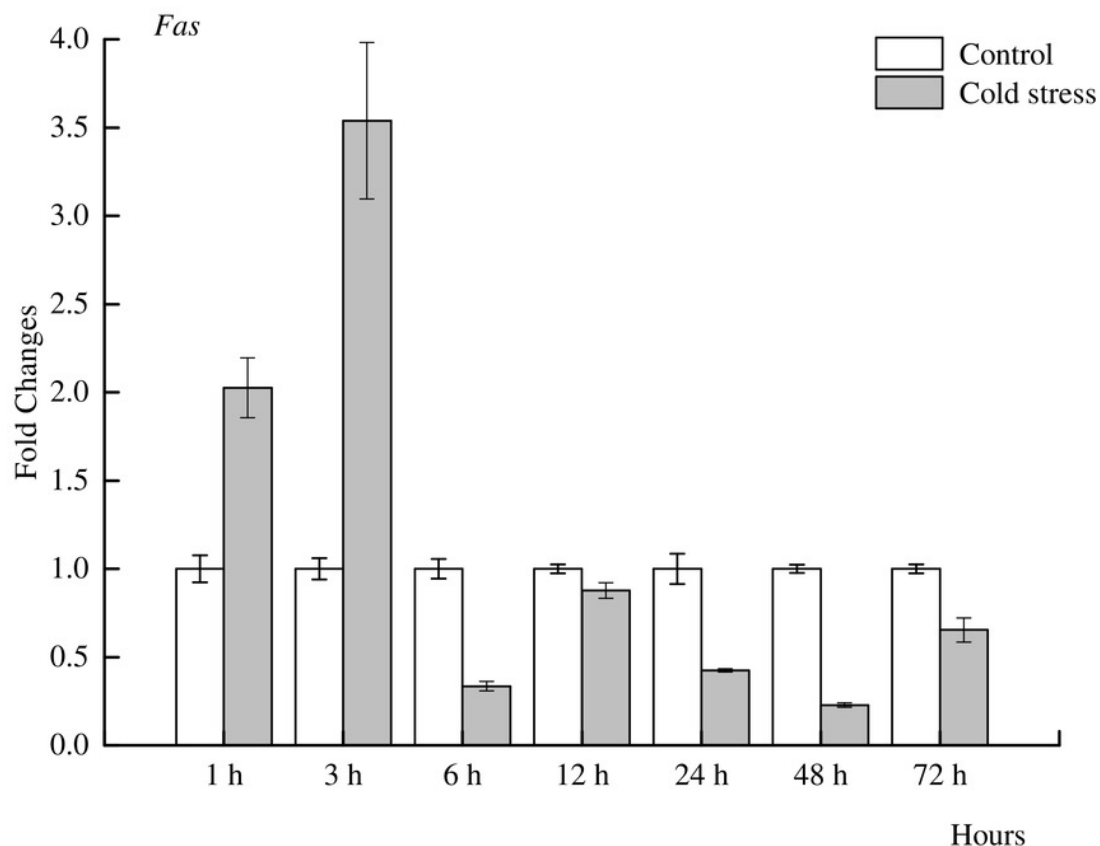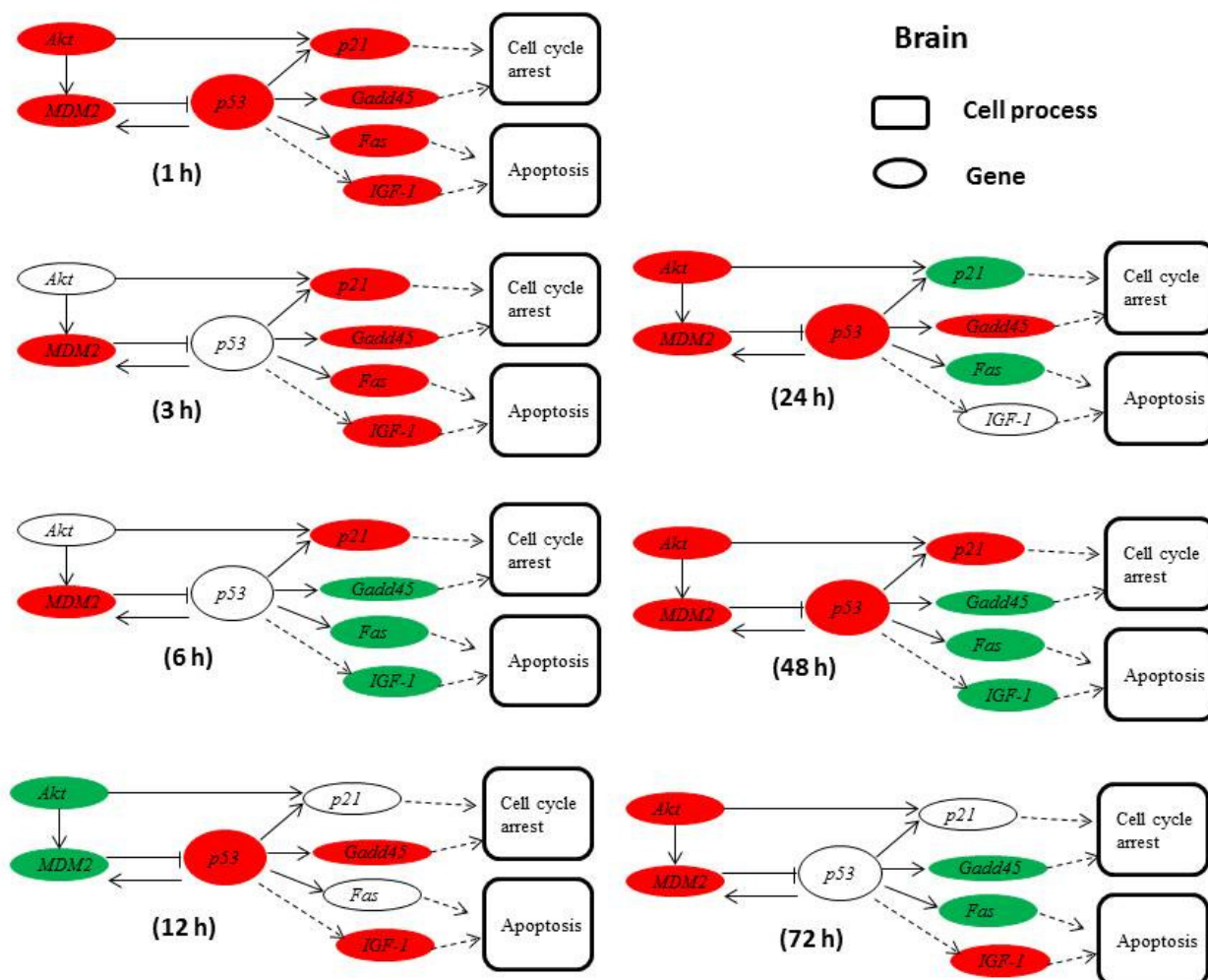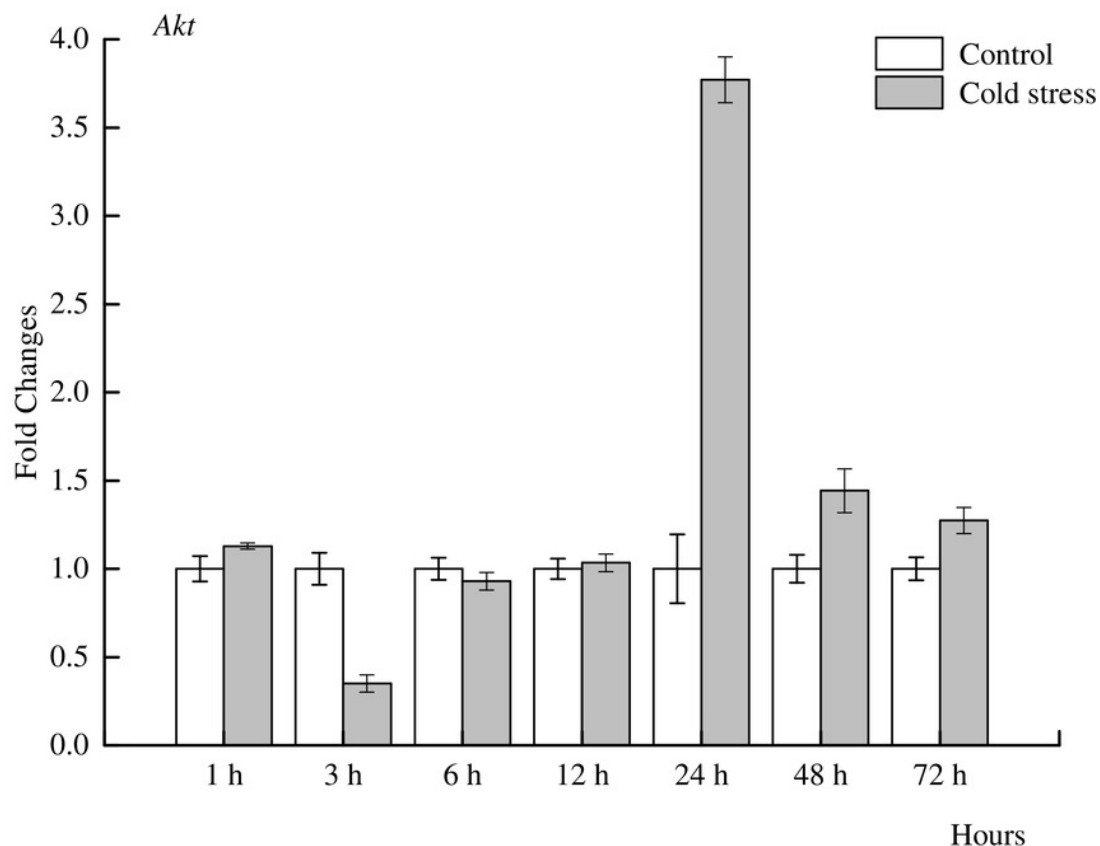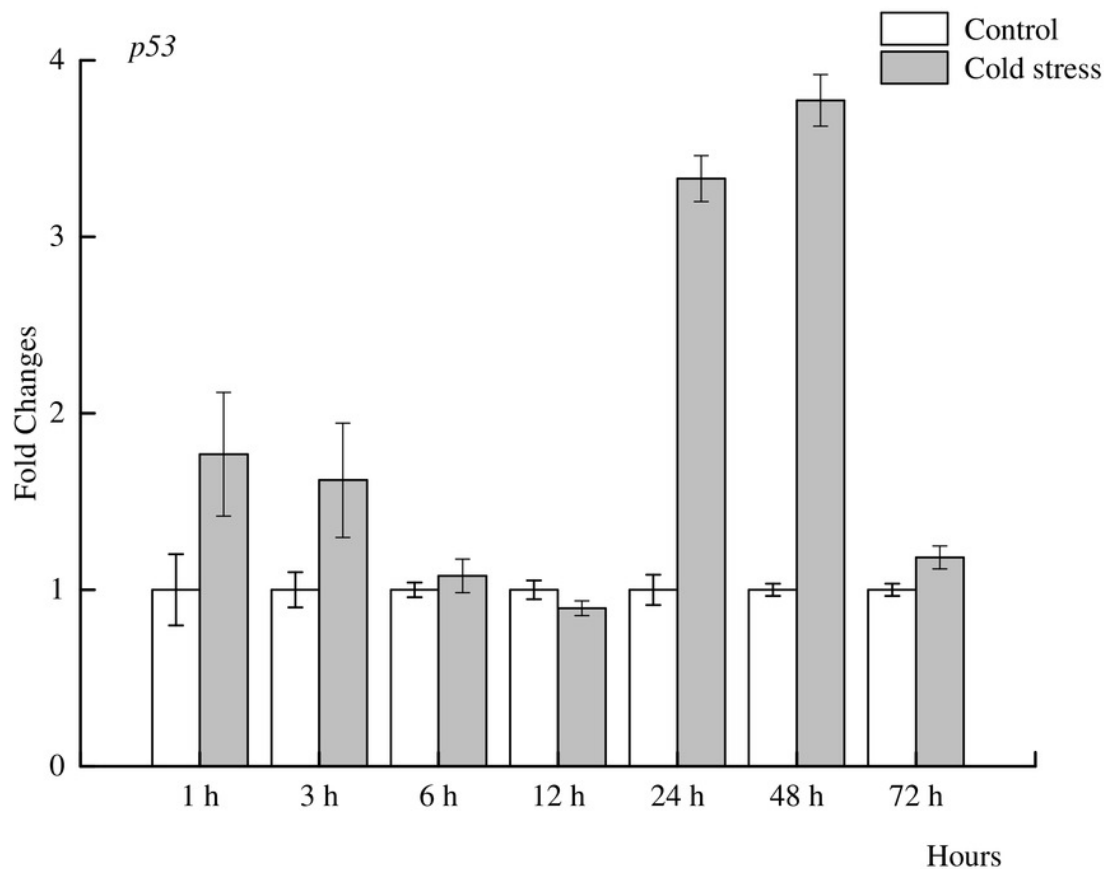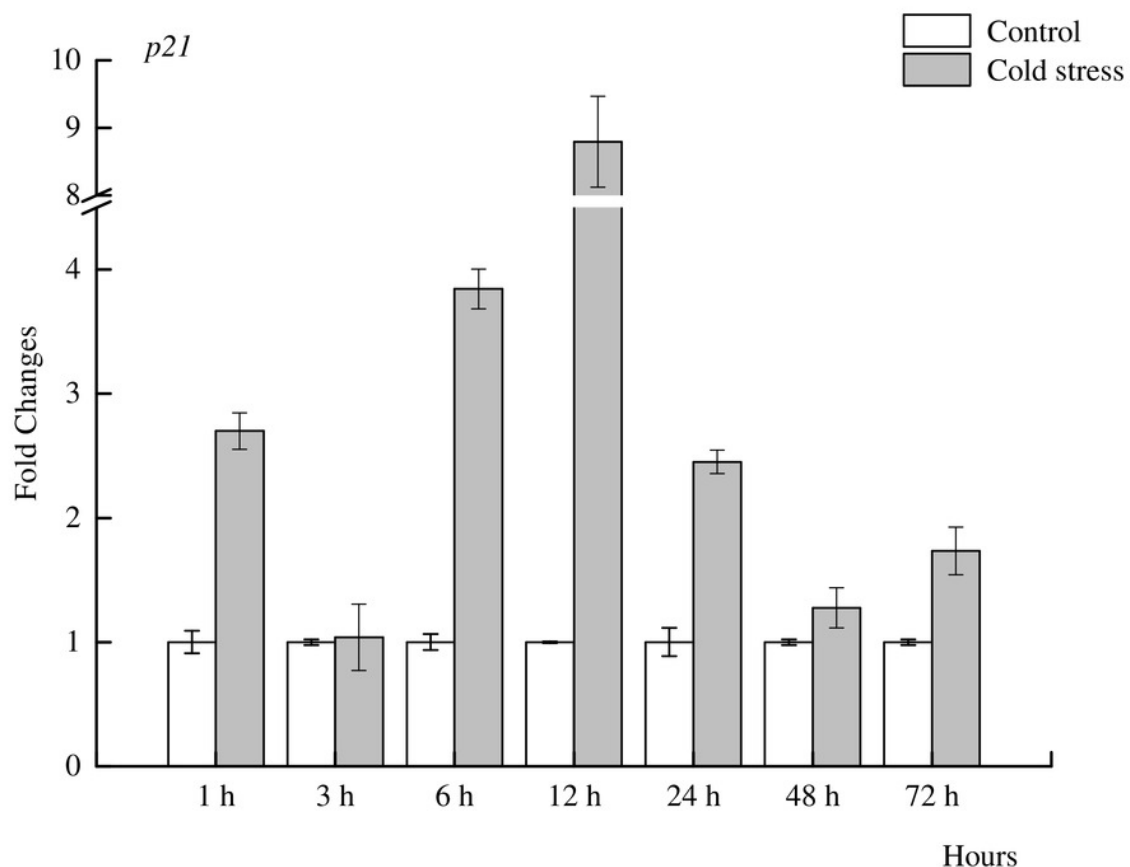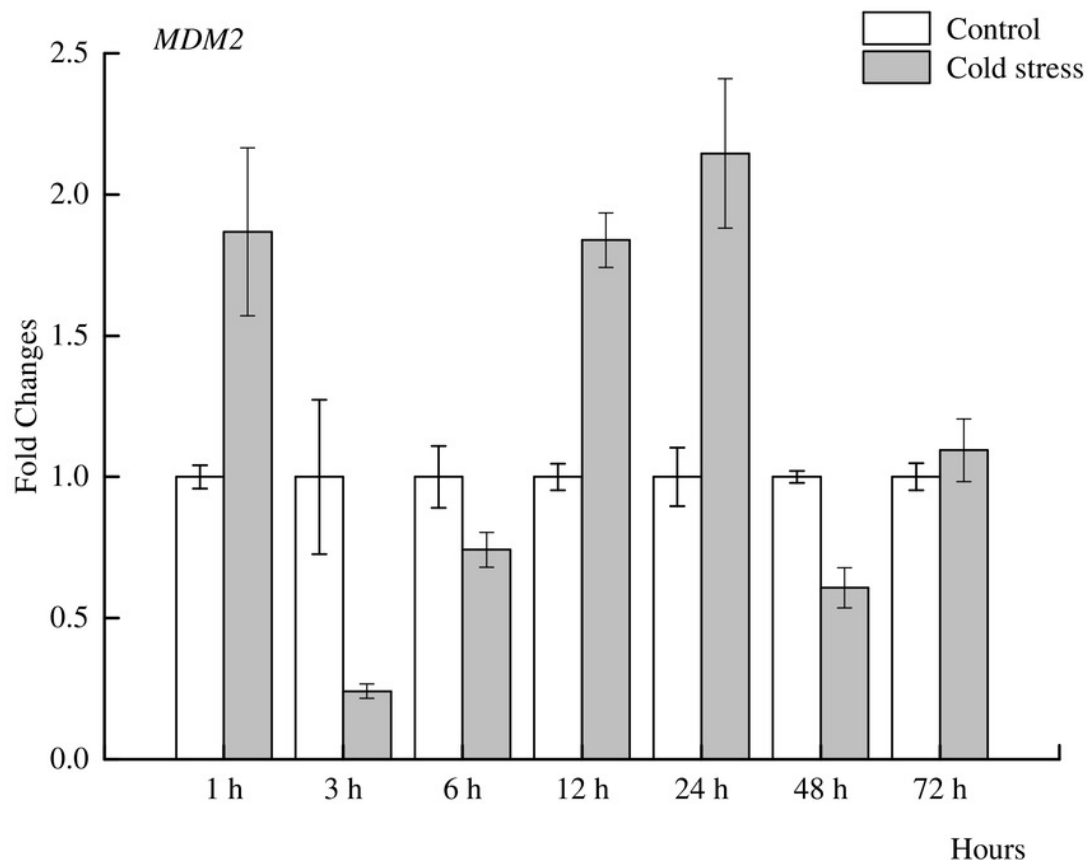410   point (1, 3, 6, 12, 24, 72 h) (Fig. 9a$_{1-7}$); putative gene networks based on qPCR datas (Fig. 9b).
411
412   **Table 1**
413   Primers for quantitative real time PCR.

414

Abbreviations:

416   p53: tumor protein p53

417   Akt: protein kinase B

418   IGF-1: insulin-like growth factor 1

419   CytC: cytochrome c

420   Apaf-1: apoptotic protease-activating factor

421   CASP8: caspase 8

422   CHK1: serine/threonine-protein kinase

423   Siah: E3 ubiquitin-protein ligase SIAH1

424   Bax: apoptosis regulator BAX

425   Fas: tumor necrosis factor receptor superfamily member 6

426   PIDD: leucine-rich repeats and death domain-containing protein

427   p21: cyclin-dependent kinase inhibitor 1A

428   CDK4/6: cyclin-dependent kinase 4/6

429   Gadd45: growth arrest and DNA damage-inducible protein

430   P53R2: ribonucleoside-diphosphate reductase subunit M2

431   Sestrins: sestrin 1/3

432   PAI: plasminogen activator inhibitor 1

433   BAI-1: adhesion G protein-coupled receptor B1

434   PTEN: phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase and dual-specificity protein phosphatase PTEN

435   TSC2: tuberous sclerosis 2

436   MDM2: E3 ubiquitin-protein ligase mdm2

437   Siah-1: E3 ubiquitin-protein ligase SIAH1

438   Cyclin G: cyclin G1

439   PIRH-2: RING finger and CHY zinc finger domain-containing protein 1

440   Cop-1: E3 ubiquitin-protein ligase RFWD2

441

Figure 1

Putative gene networks in large yellow croaker stressed by 12 h acute cold based on the transcriptome data of previous studies.

Putative gene networks in large yellow croaker stressed by 12 h acute cold based on the transcriptome data of previous studies. Enriched gene networks associated with cell cycle arrest, apoptosis, p53 negative feedback, and DNA repair and damage prevention. Red indicates up-regulated, blue down-regulated, white indicates no changes. Full names of abbreviated genes are listed in Abbreviation.

Figure 2

qPCR analysis of *Akt* in the liver of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

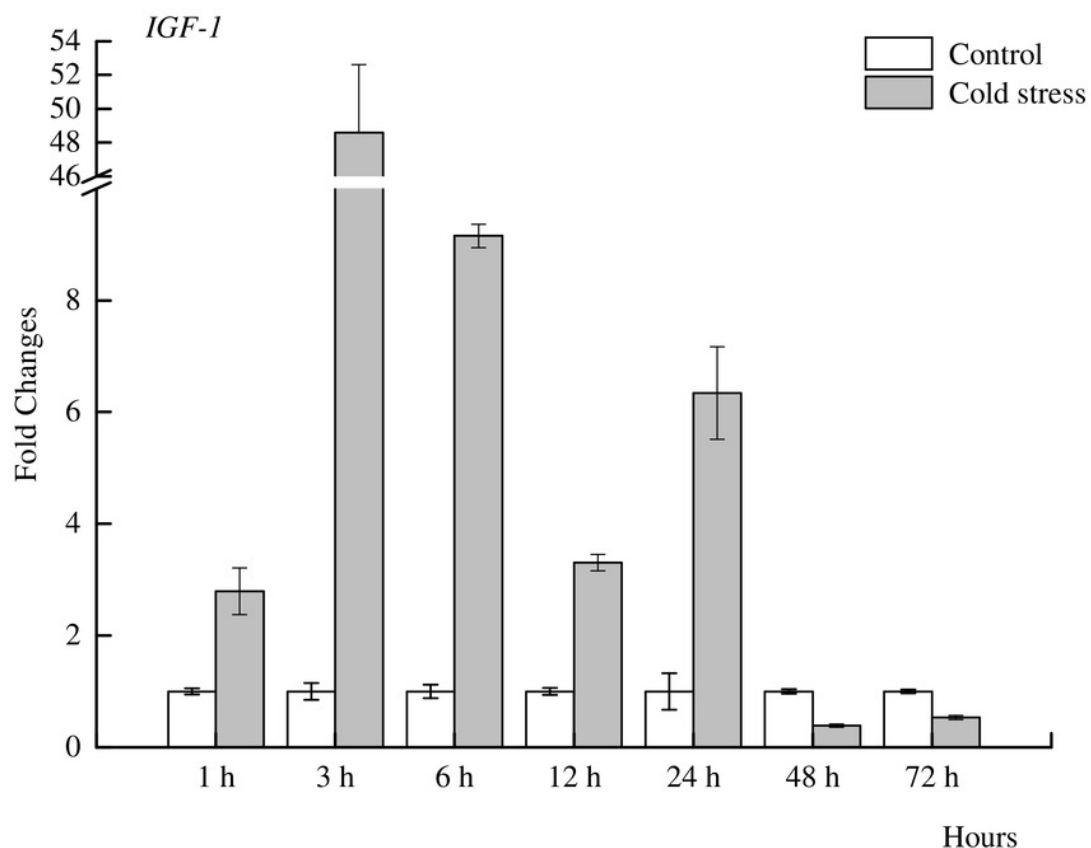# Figure 3

qPCR analysis of *p53* in the liver of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
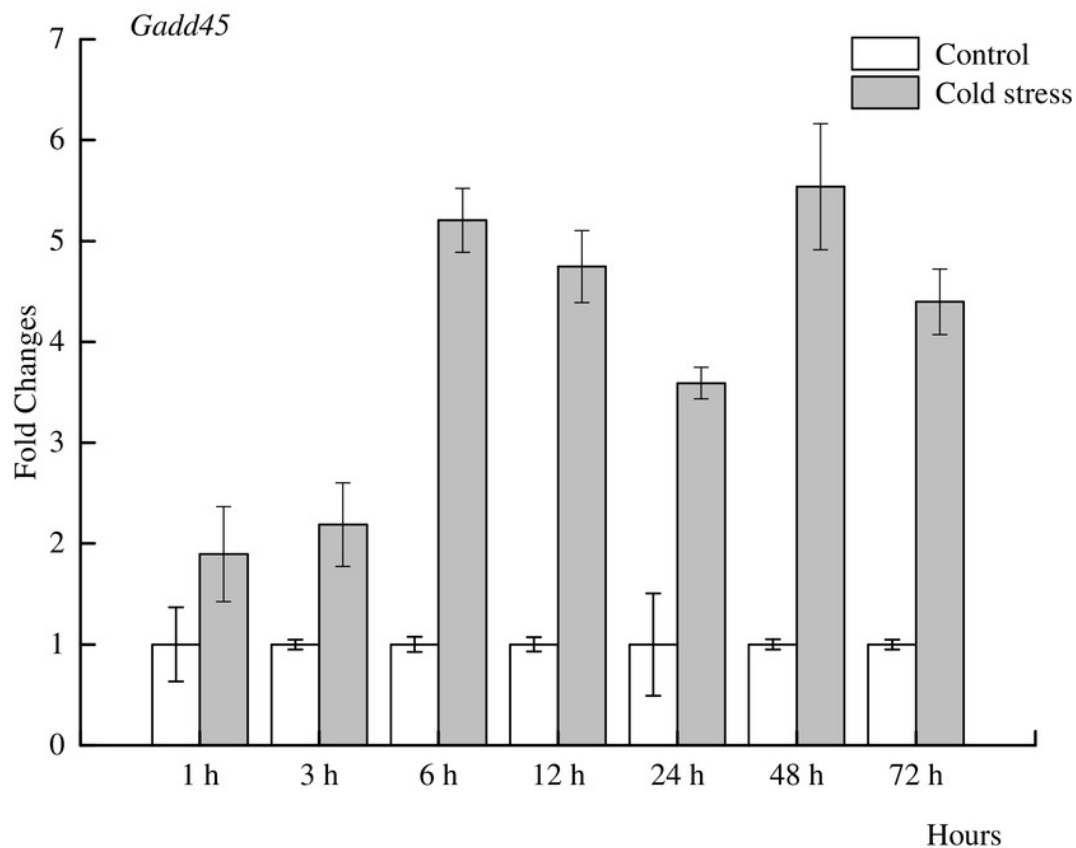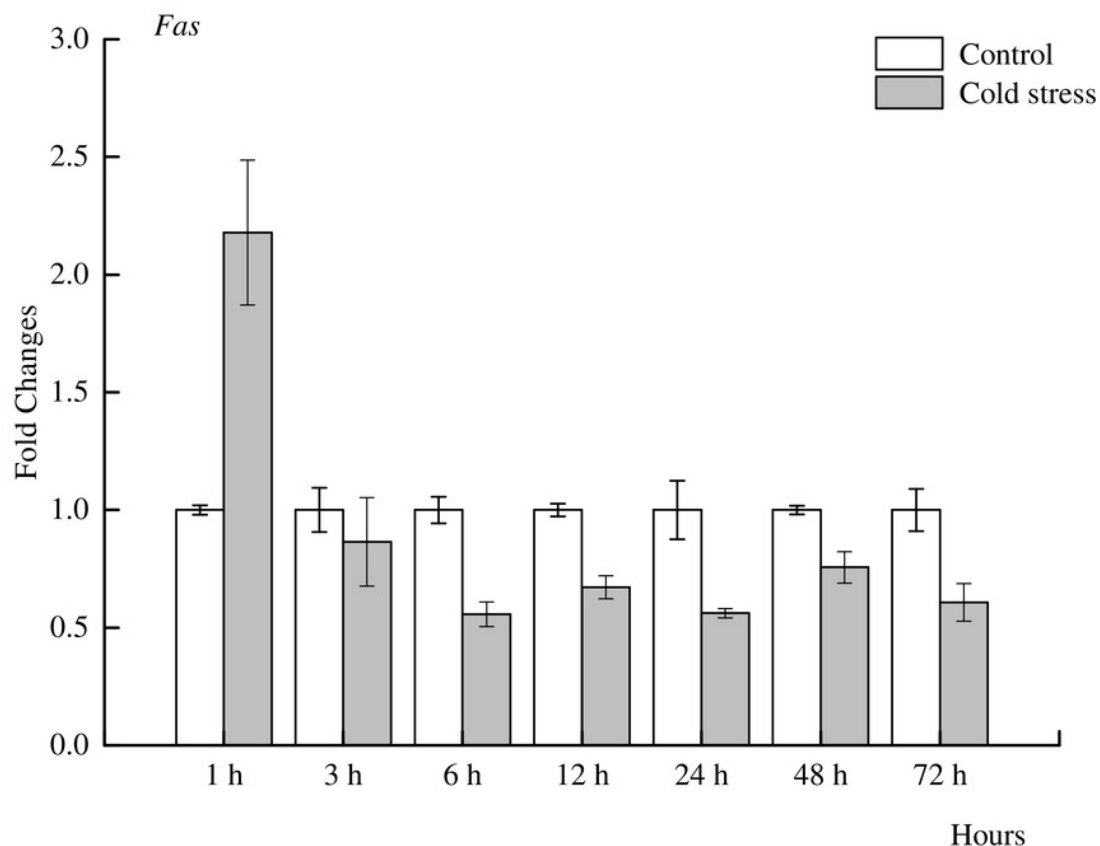
Figure 4

qPCR analysis of *p21* in the liver of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 5

qPCR analysis of *MDM2* in the liver of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
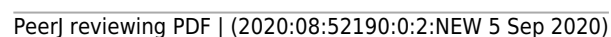
Figure 6

qPCR analysis of *IGF-1* in the liver of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 7

qPCR analysis of *Gadd45* in the liver of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
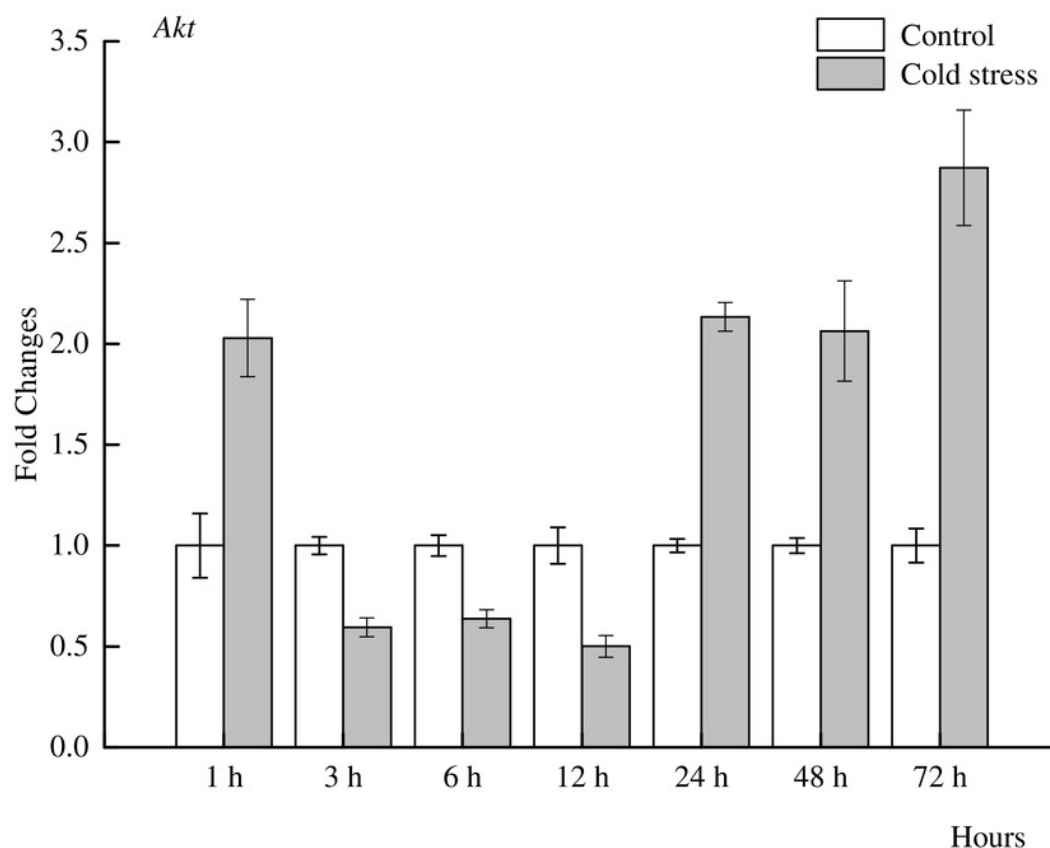
Figure 8

qPCR analysis of *Fas* in the liver of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
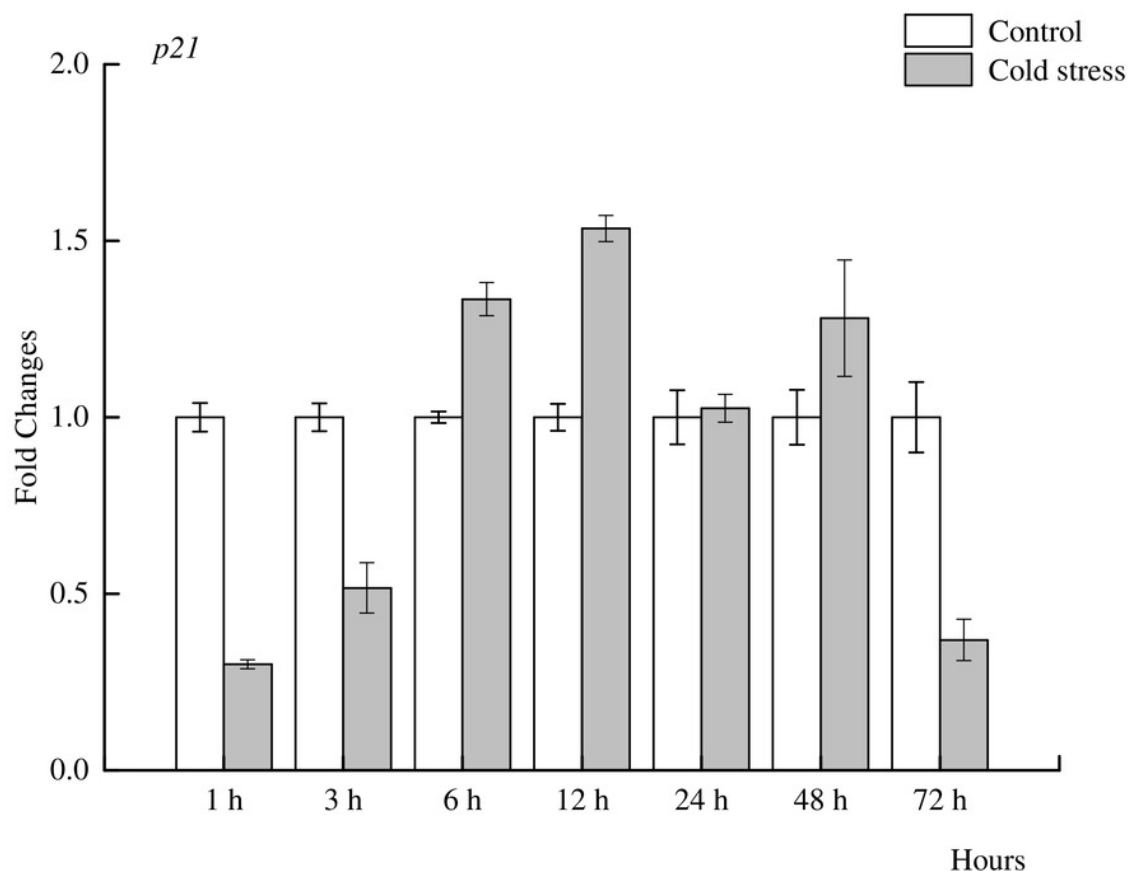
Figure 9

putative gene networks based on qPCR datas in liver

Figure 10

qPCR analysis of *Akt* in the muscle of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 11

qPCR analysis of *p53* in the muscle of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
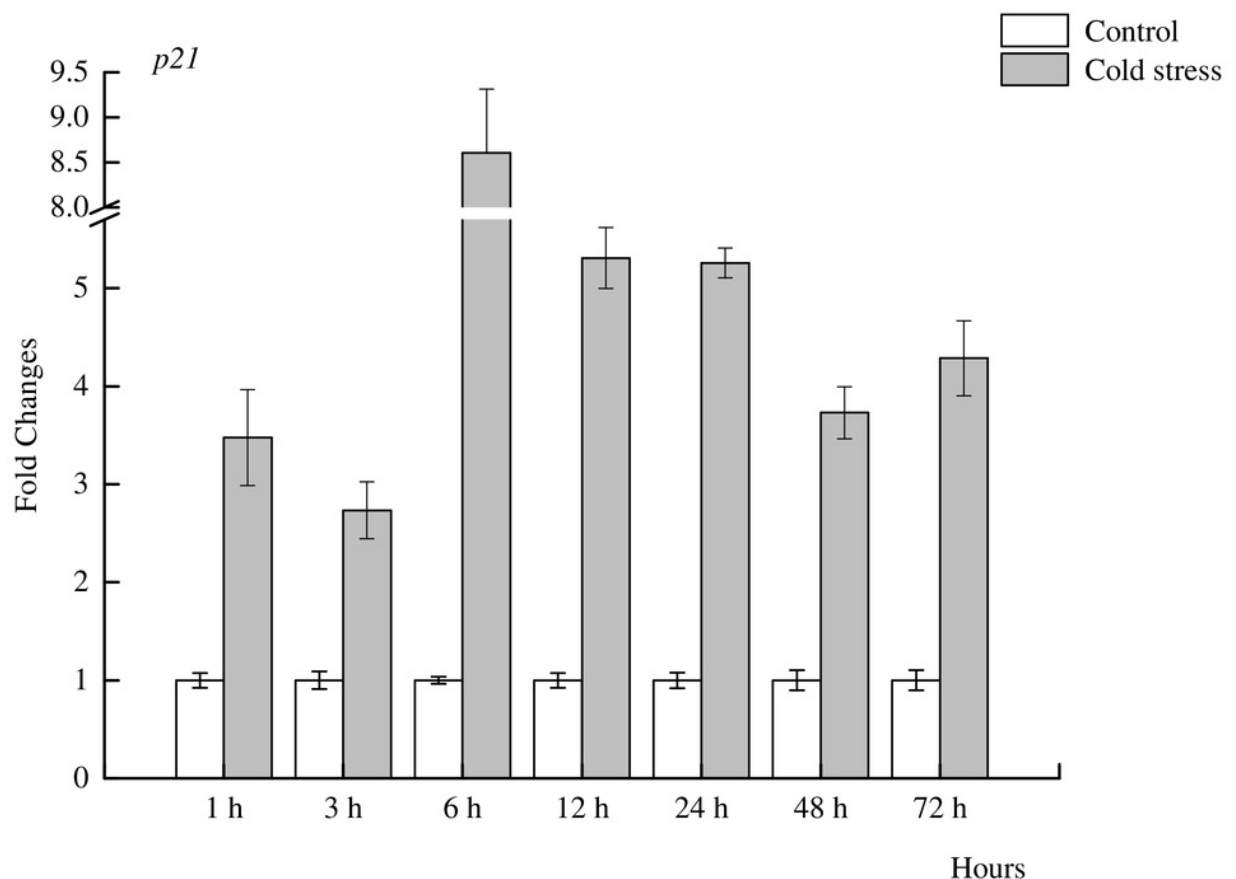
Figure 12

qPCR analysis of *MDM2* in the muscle of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 13

qPCR analysis of *p21* in the muscle of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
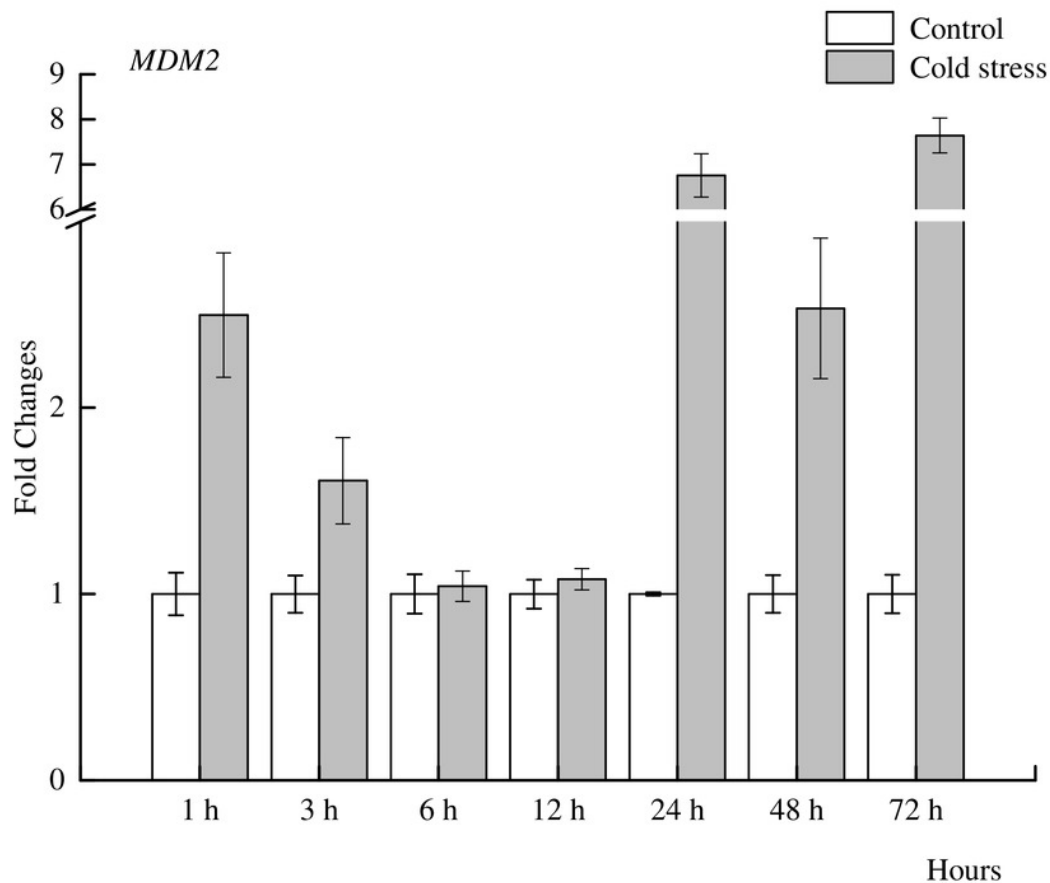
Figure 14

qPCR analysis of *IGF-1* in the muscle of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
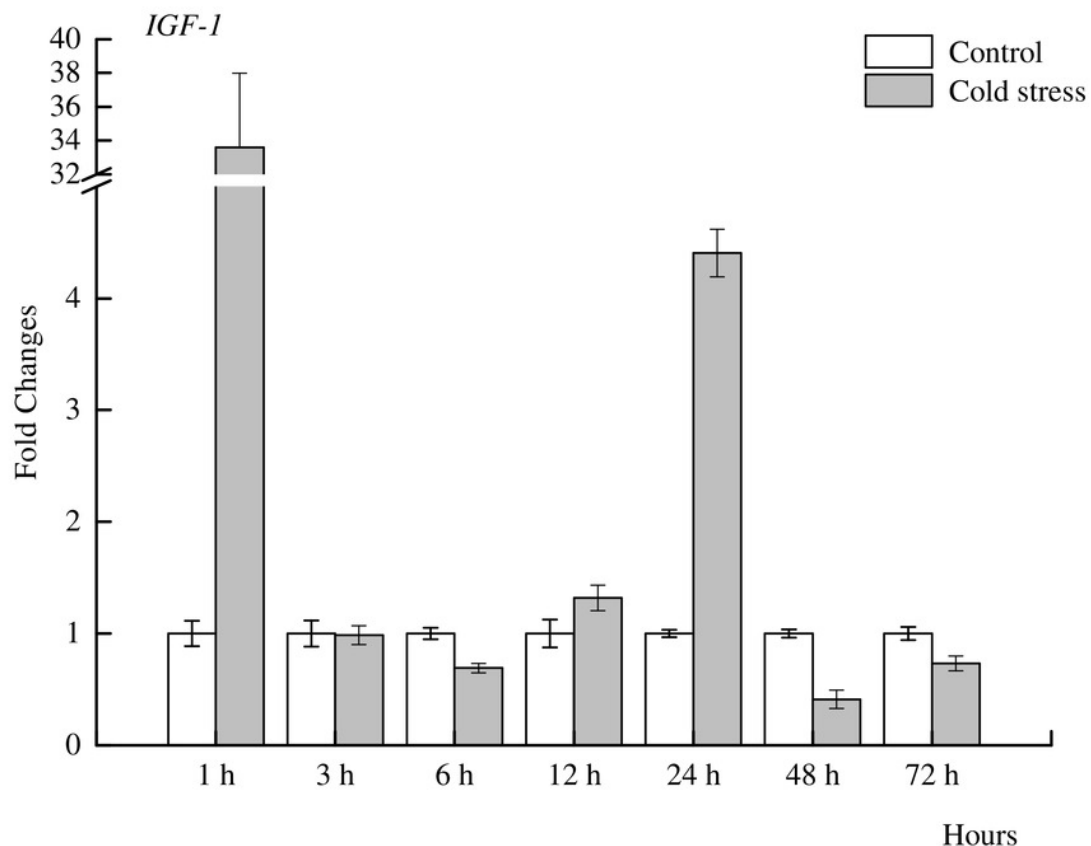
Figure 15

qPCR analysis of *Gadd45* in the muscle of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 16

qPCR analysis of *Fas* in the muscle of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 17

putative gene networks based on qPCR datas in muscle

Figure 18

qPCR analysis of *Akt* in the brain of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 19

qPCR analysis of *p53* in the brain of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
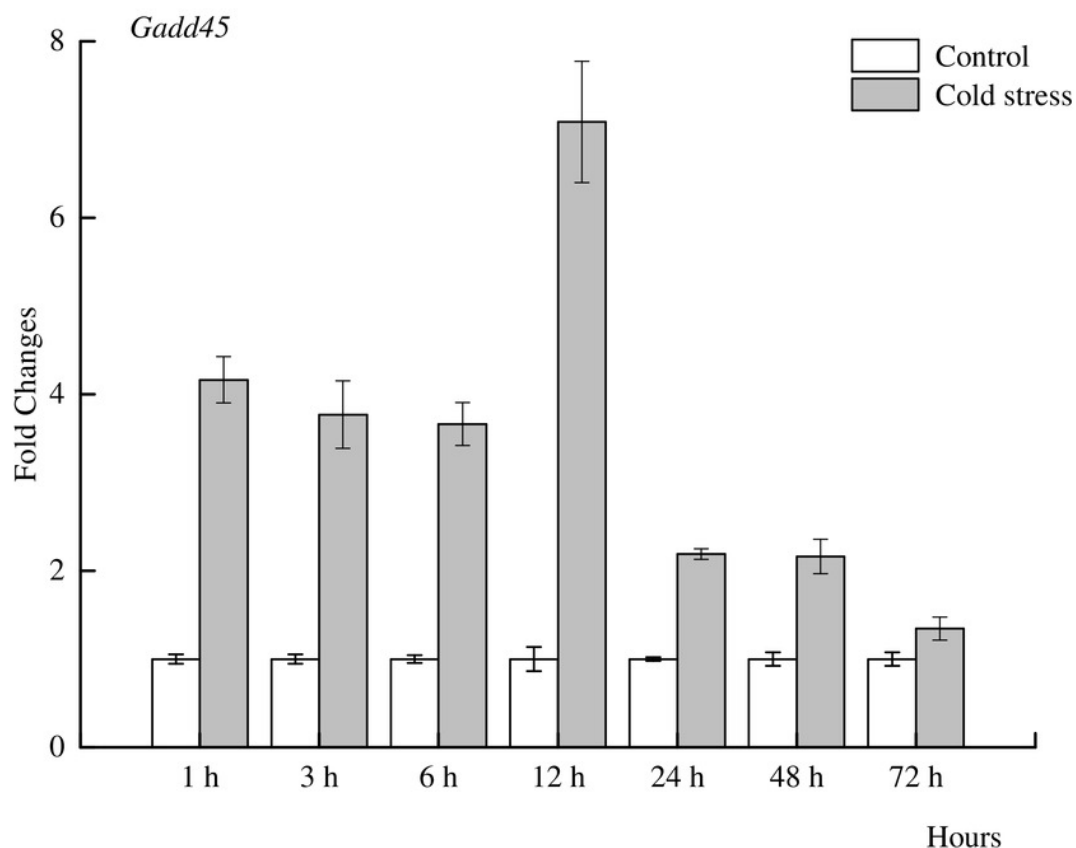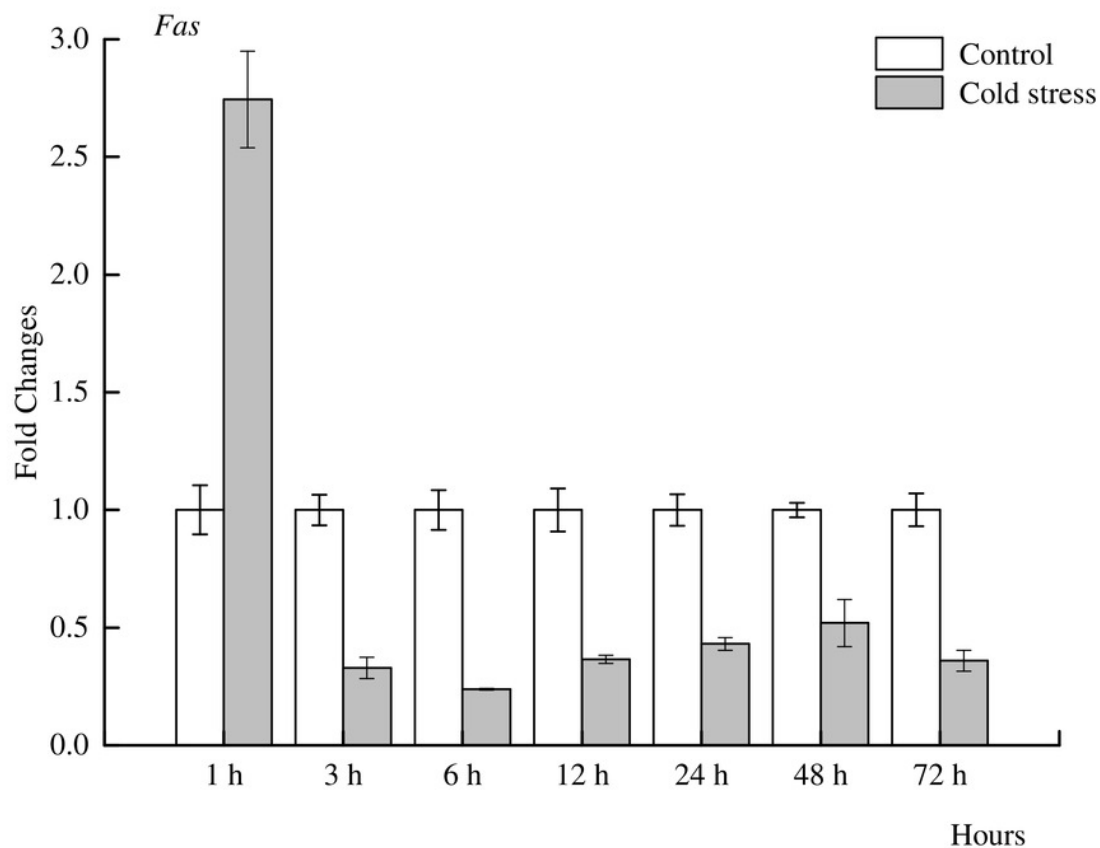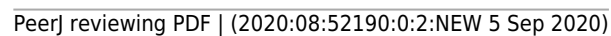
Figure 20

qPCR analysis of *p21* in the brain of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 21

qPCR analysis of *MDM2* in the brain of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 22

qPCR analysis of *IGF-1* in the brain of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 23

qPCR analysis of *Gadd45* in the brain of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 24

qPCR analysis of *Fas* in the brain of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
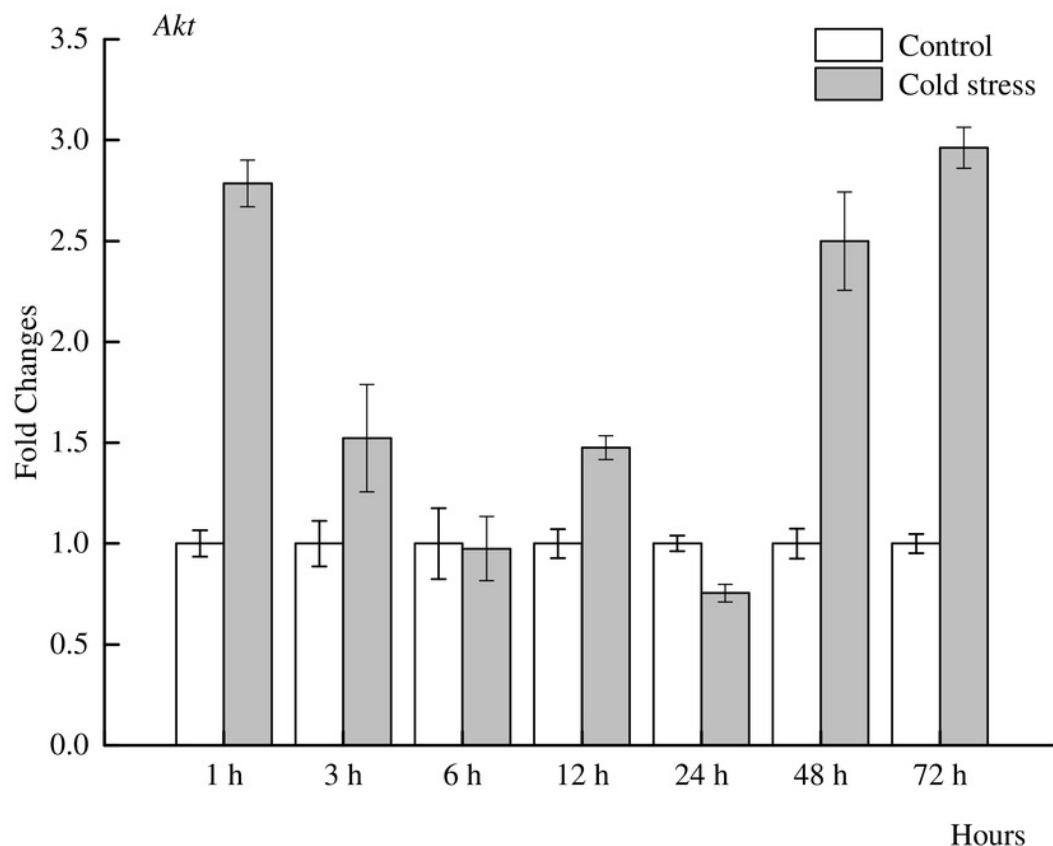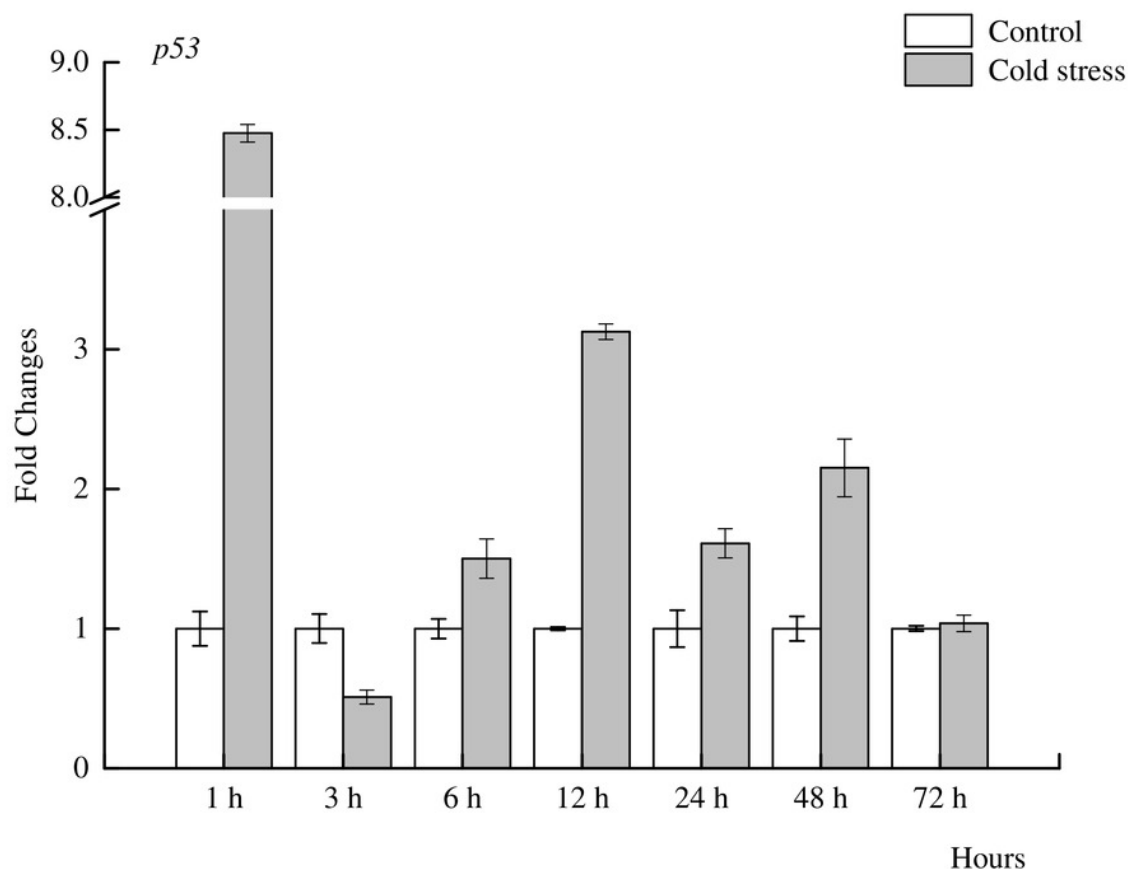
Figure 25

putative gene networks based on qPCR datas in brain

Figure 26

qPCR analysis of *Akt* in the spleen of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 27

qPCR analysis of *p53* in the spleen of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 28

qPCR analysis of *p21* in the spleen of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
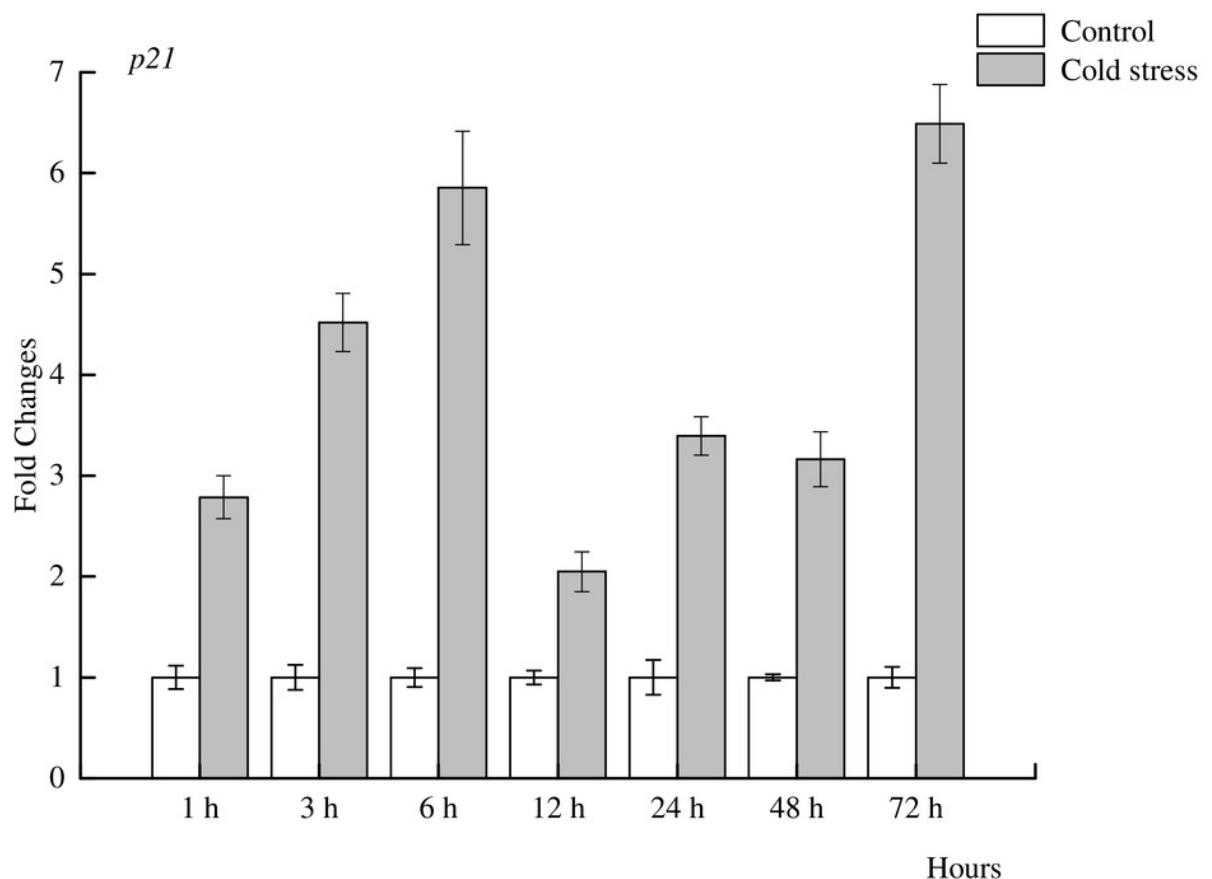
Figure 29

qPCR analysis of *MDM2* in the spleen of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
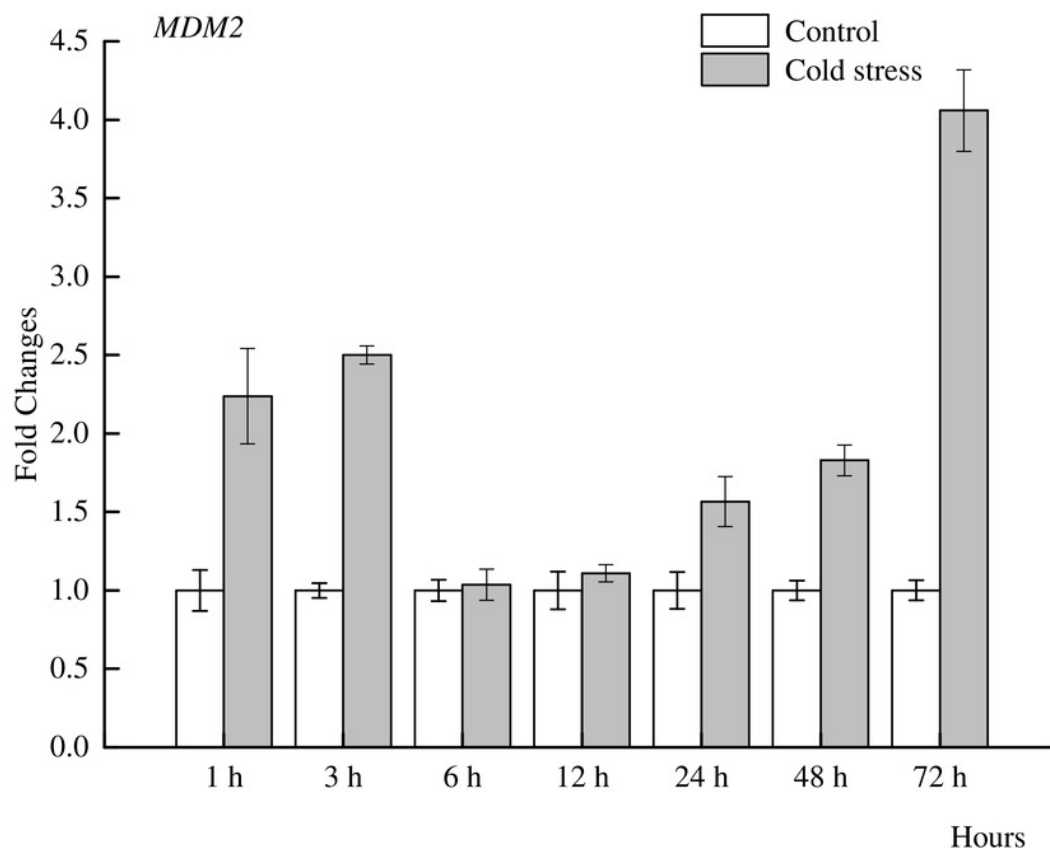
Figure 30

qPCR analysis of *IGF-1* in the spleen of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
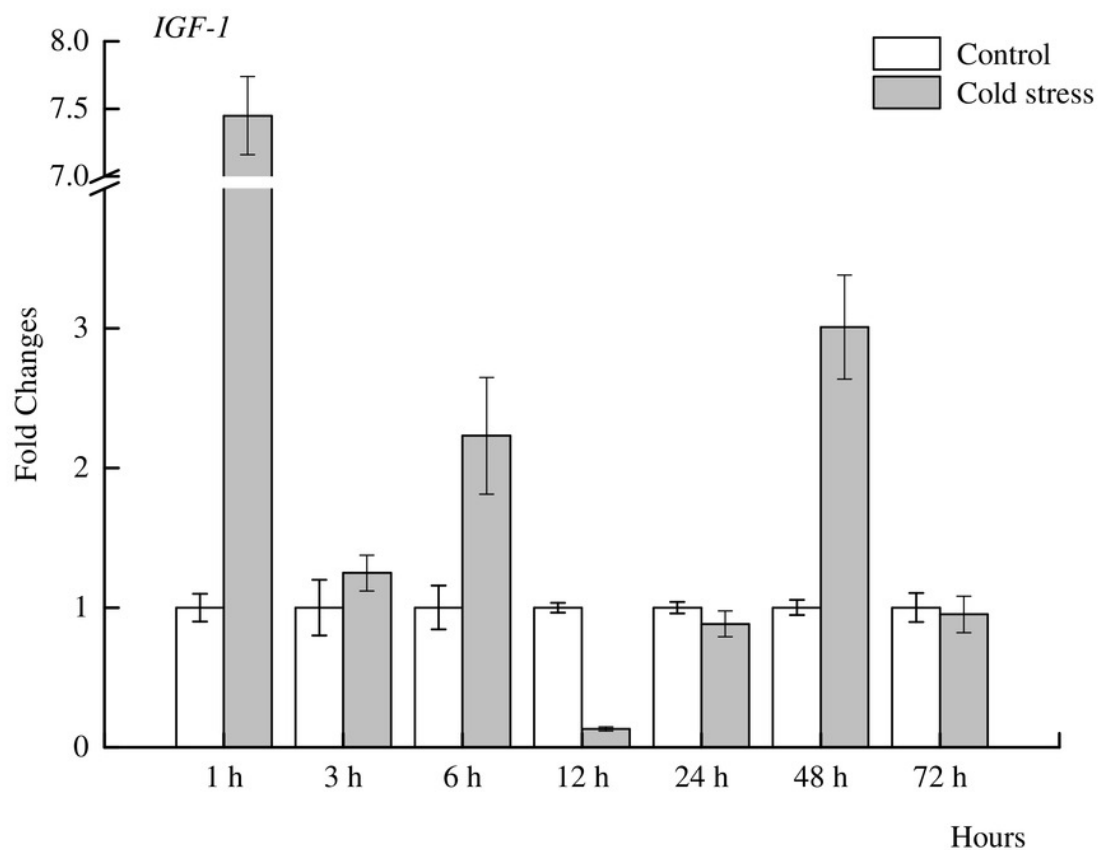
Figure 31

qPCR analysis of *Gadd45* in the spleen of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
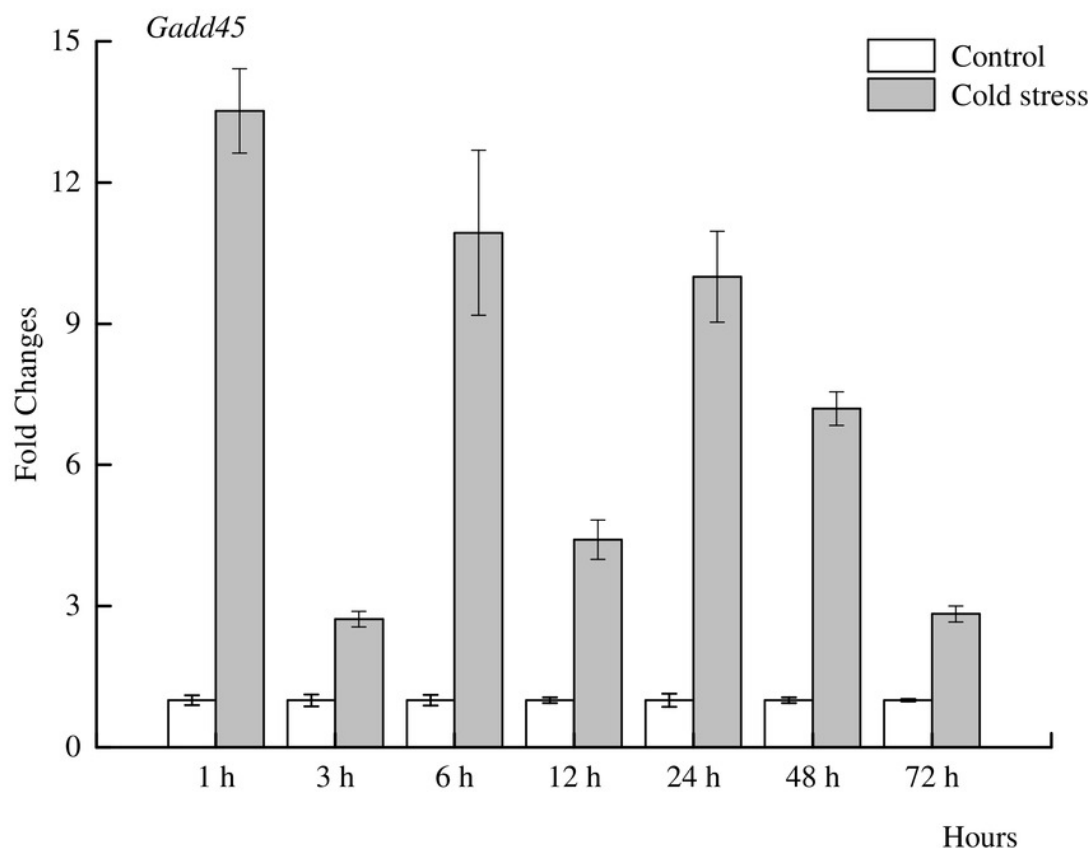
Figure 32

qPCR analysis of *Fas* in the spleen of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 33

putative gene networks based on qPCR datas in spleen

Figure 34

qPCR analysis of *Akt* in the gill of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
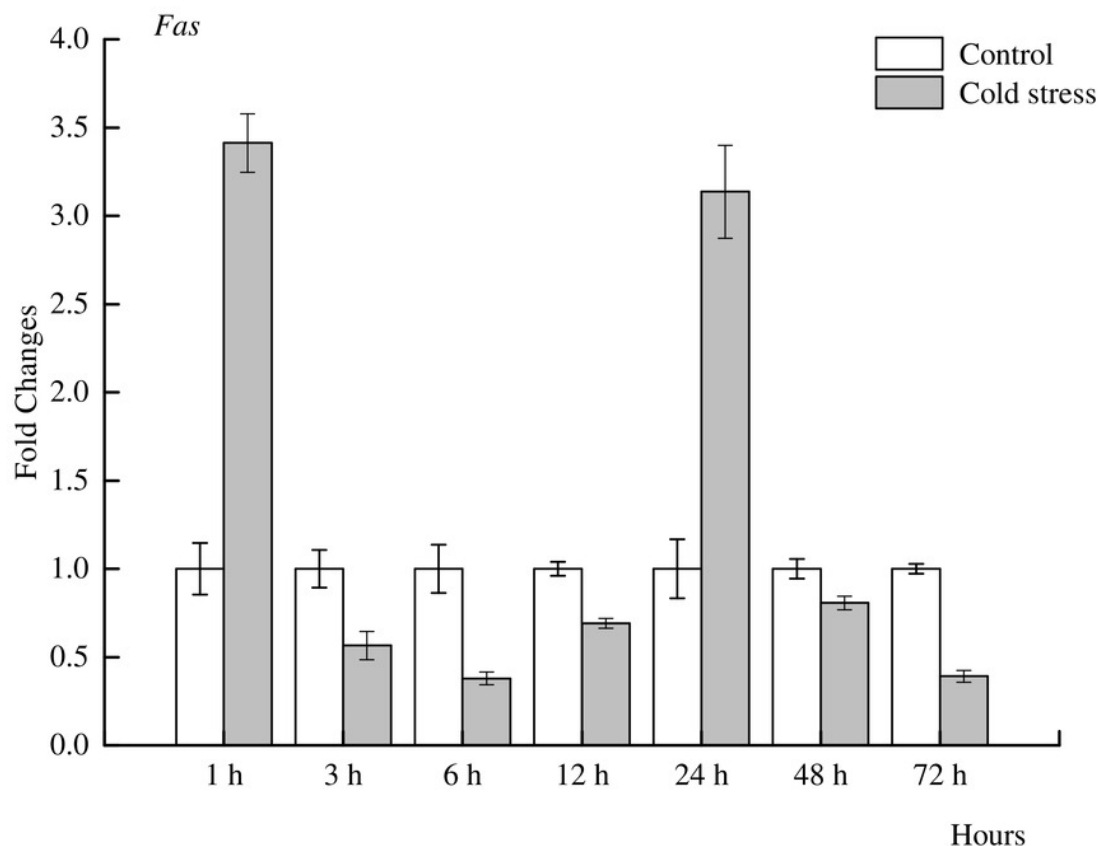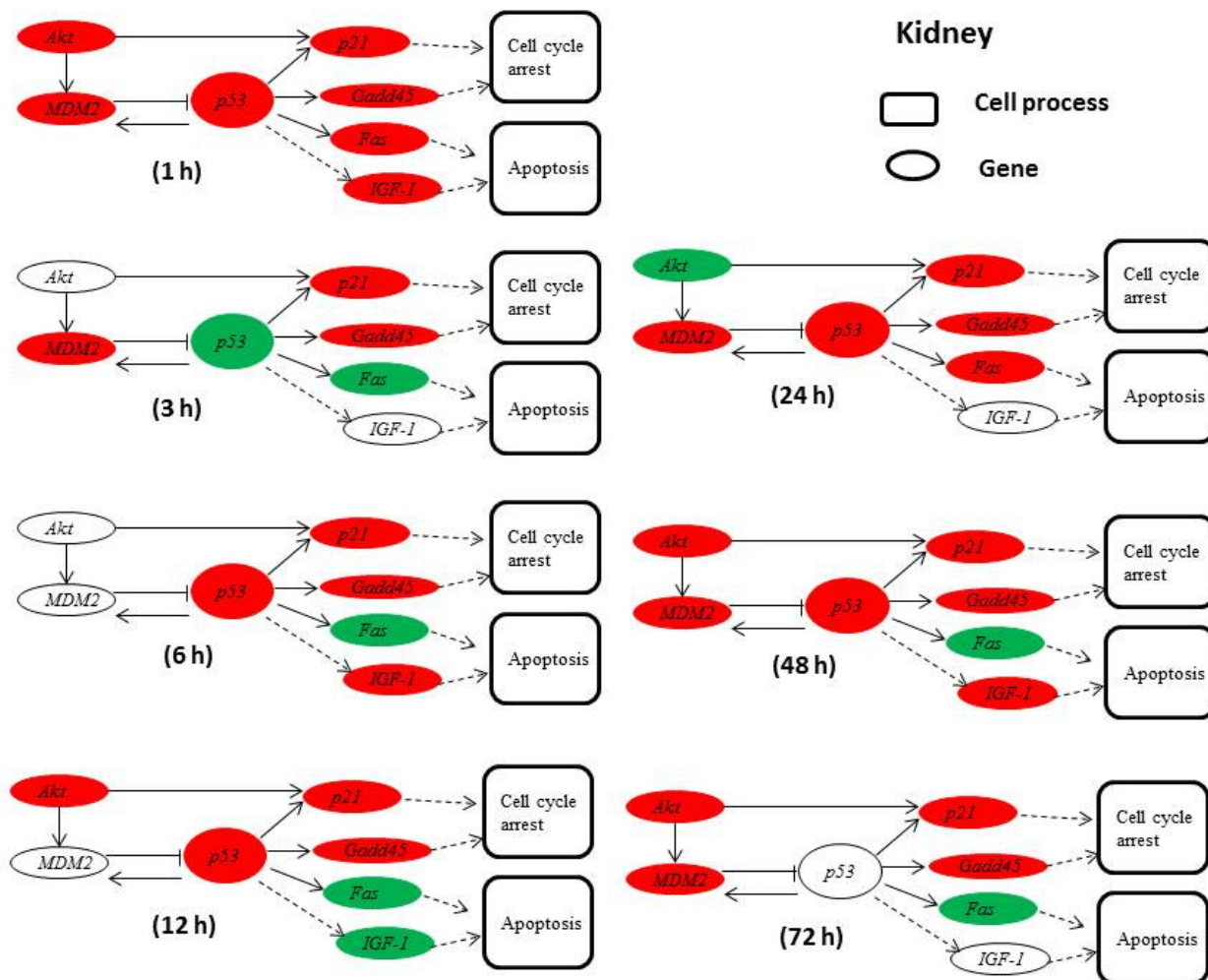
Figure 35

qPCR analysis of *p53* in the gill of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

PeerJ

Figure 36

qPCR analysis of *p21* in the gill of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 37

qPCR analysis of *MDM2* in the gill of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 38

qPCR analysis of *IGF-1* in the gill of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 39

qPCR analysis of *Gadd45* in the gill of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 40

qPCR analysis of *Fas* in the gill of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
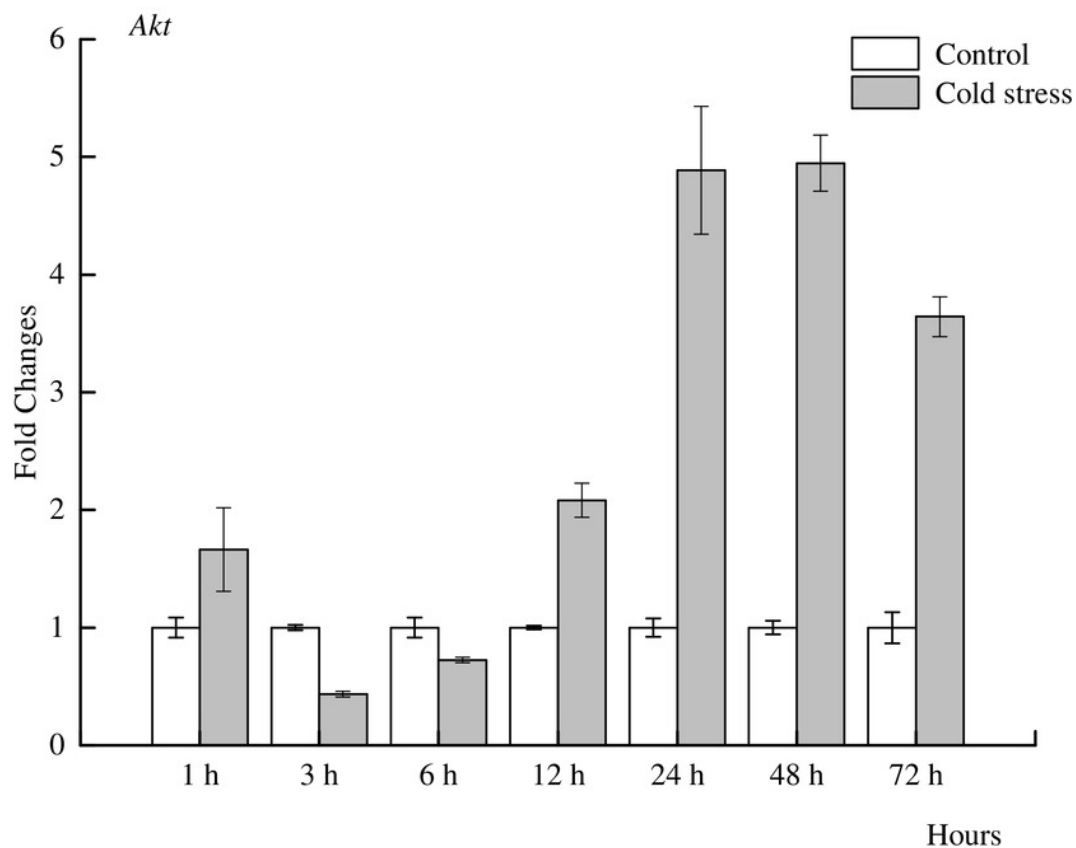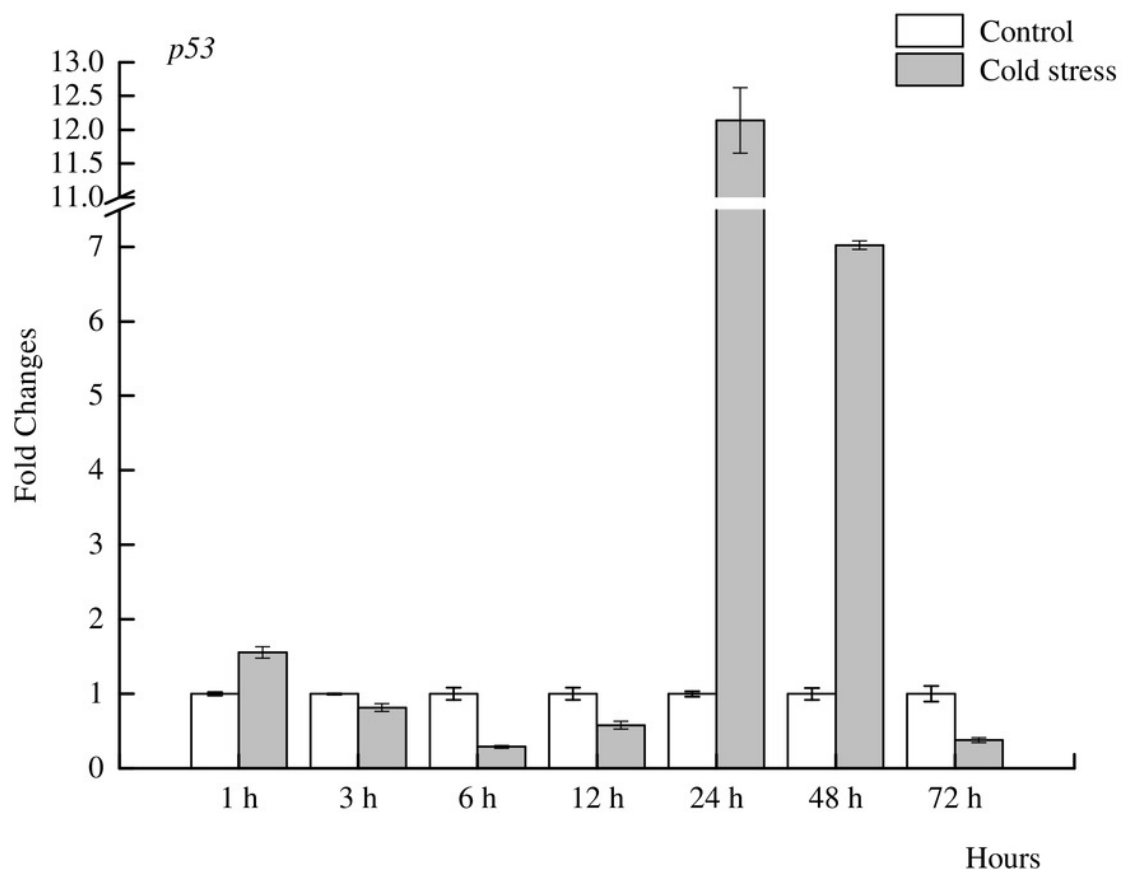
Figure 41

putative gene networks based on qPCR datas in gill

Figure 42

qPCR analysis of *Akt* in the kidney of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
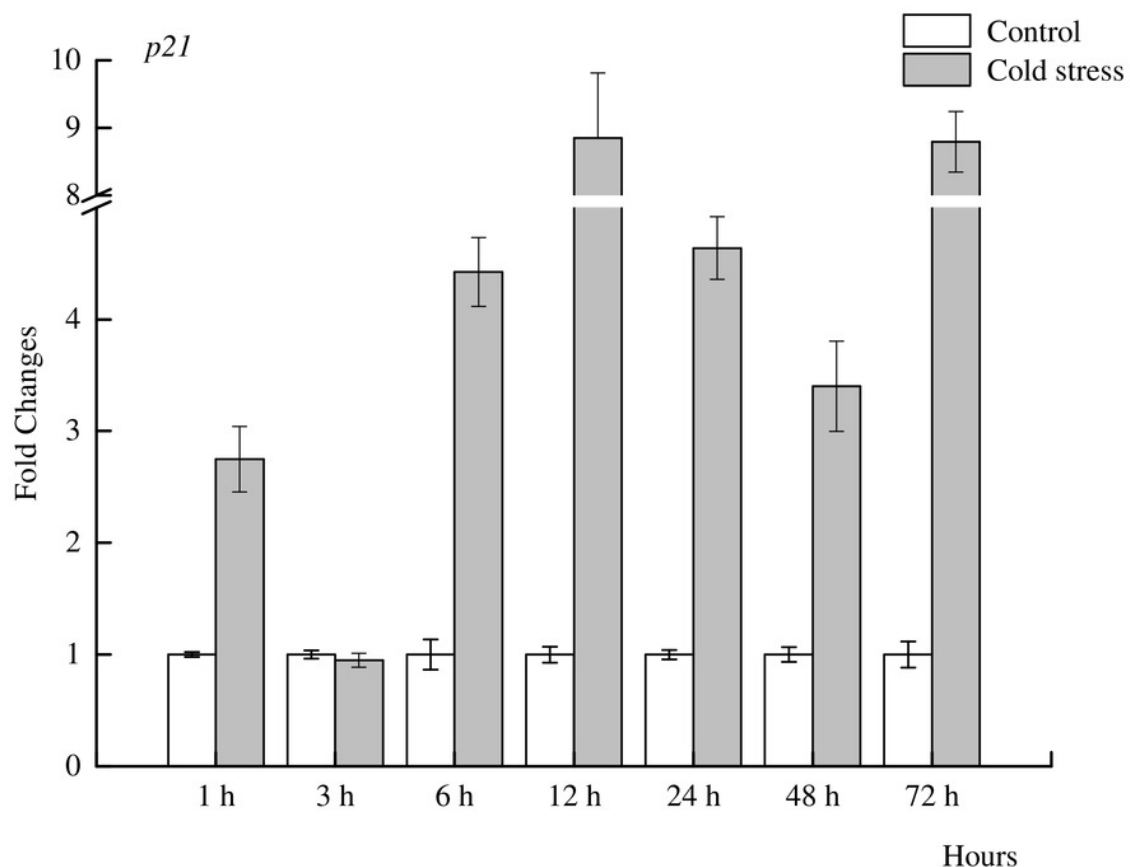
Figure 43

qPCR analysis of *p53* in the kidney of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
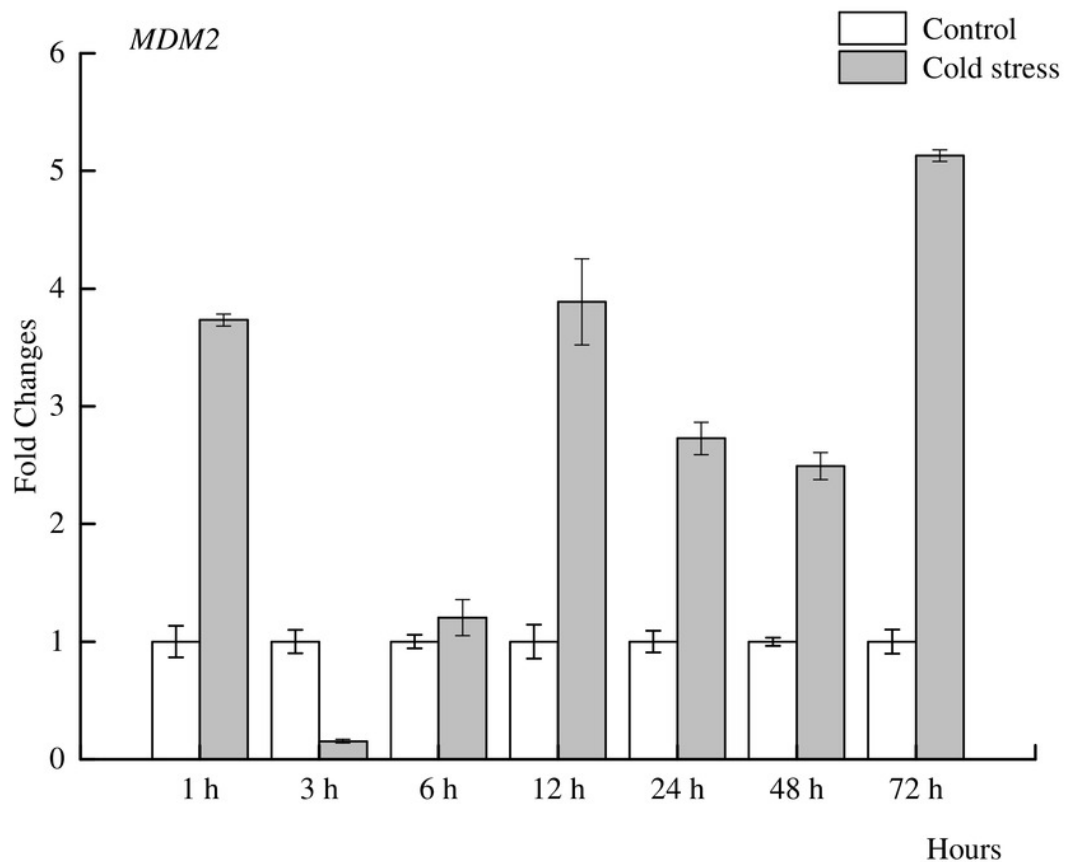
Figure 44

qPCR analysis of *p21* in the kidney of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 45

qPCR analysis of *MDM2* in the kidney of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 46

qPCR analysis of *IGF-1* in the kidney of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
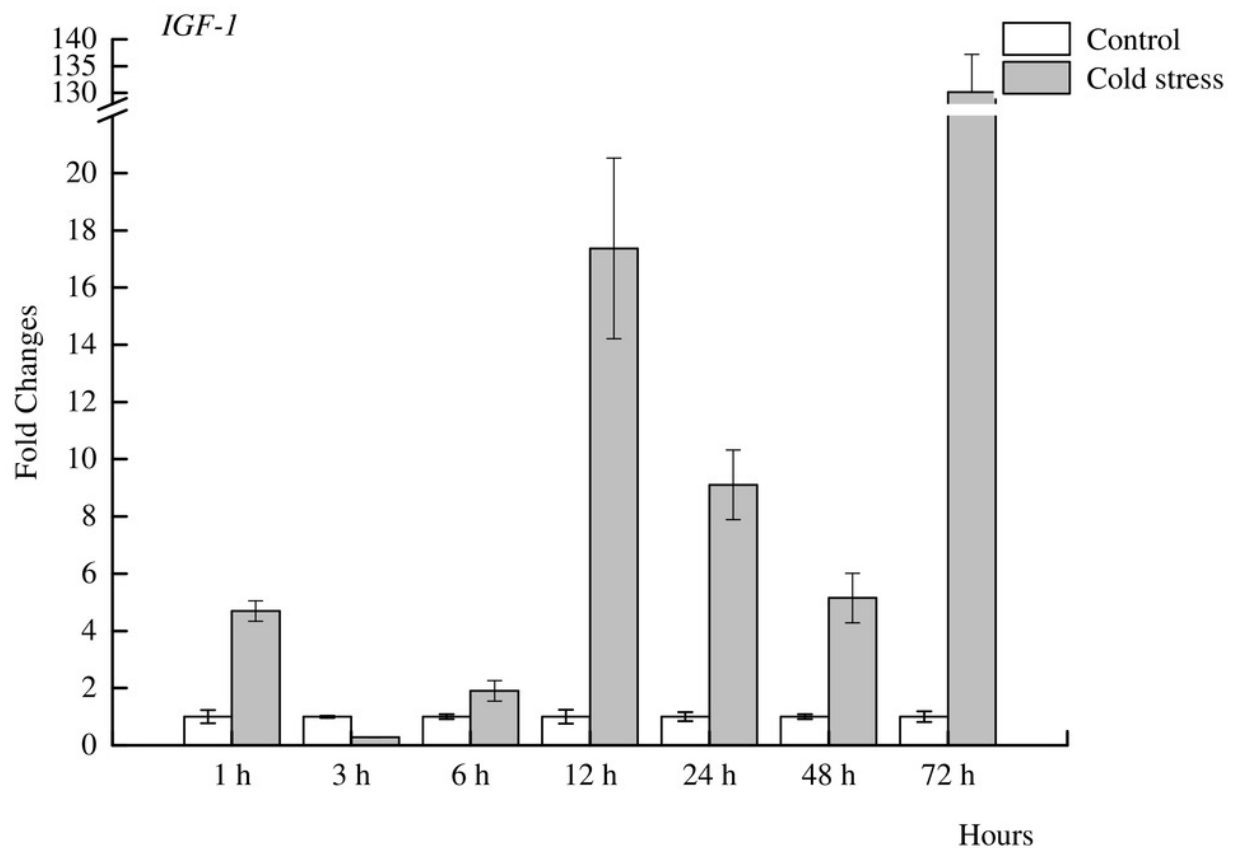
Figure 47

qPCR analysis of *Gadd45* in the kidney of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
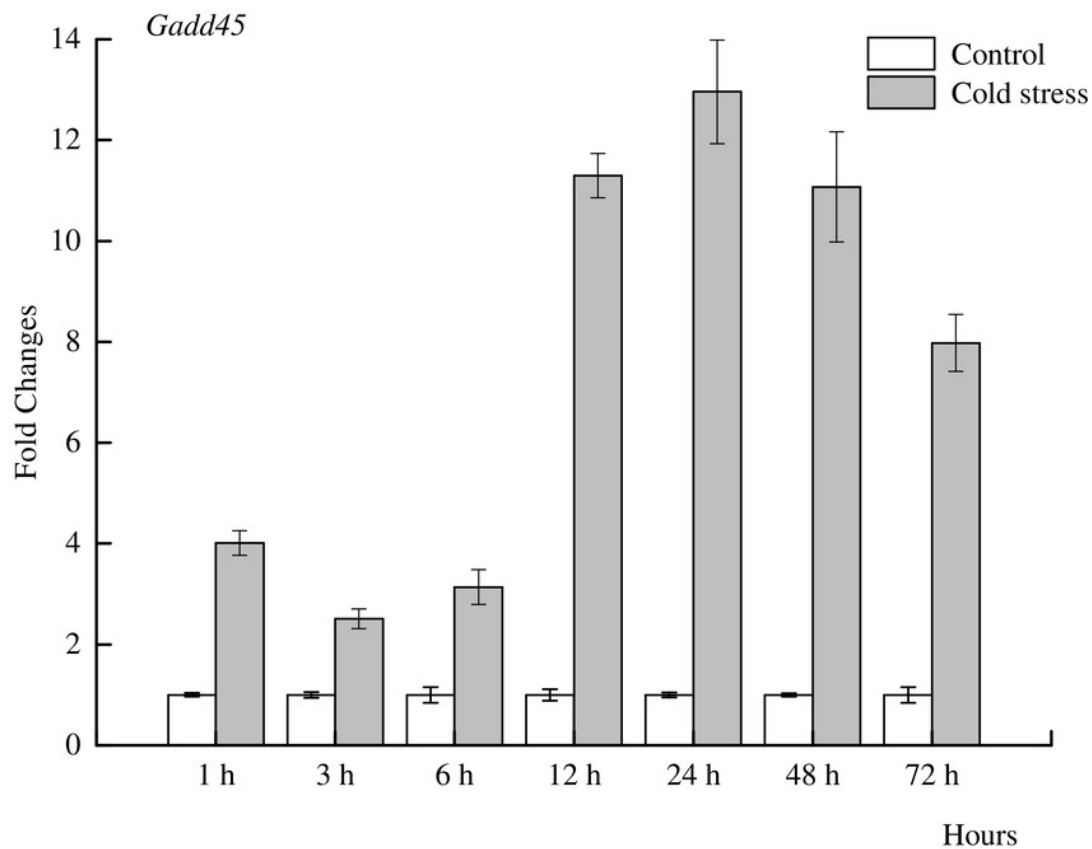
Figure 48

qPCR analysis of *Fas* in the kidney of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 49

putative gene networks based on qPCR datas in kidney

Figure 50

qPCR analysis of *Akt* in the intestine of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
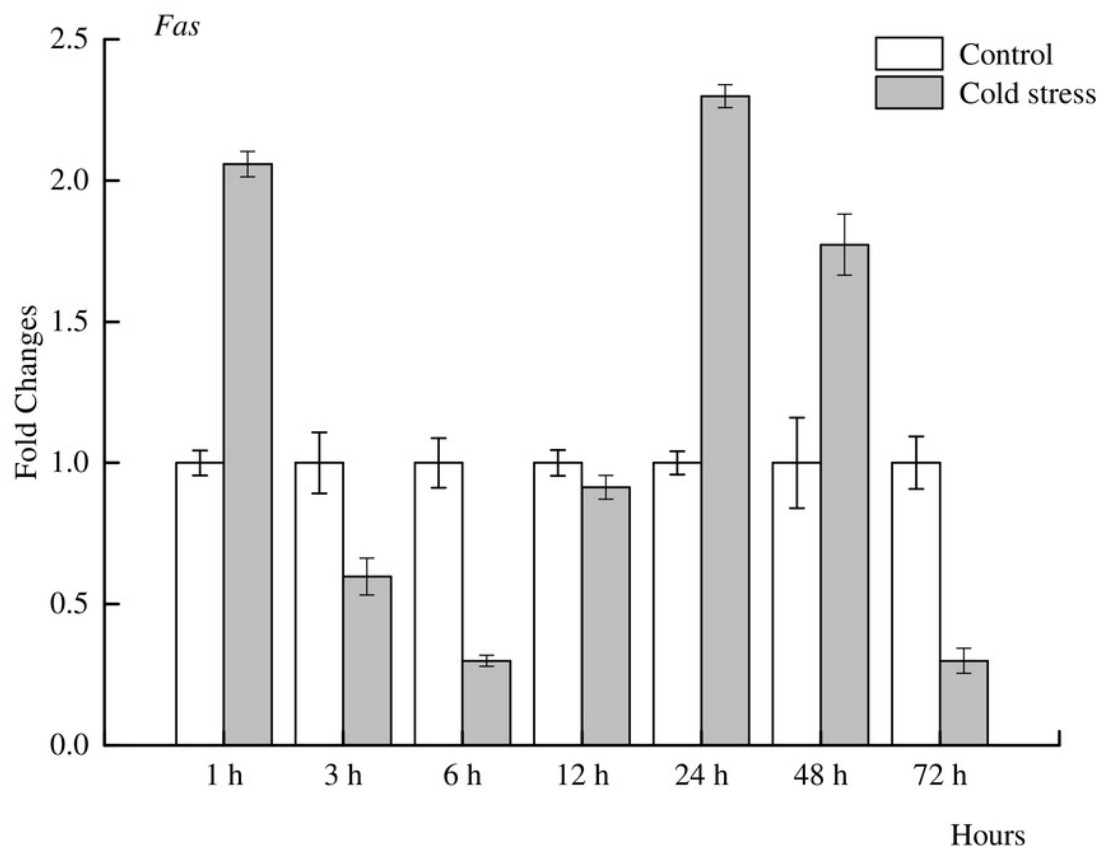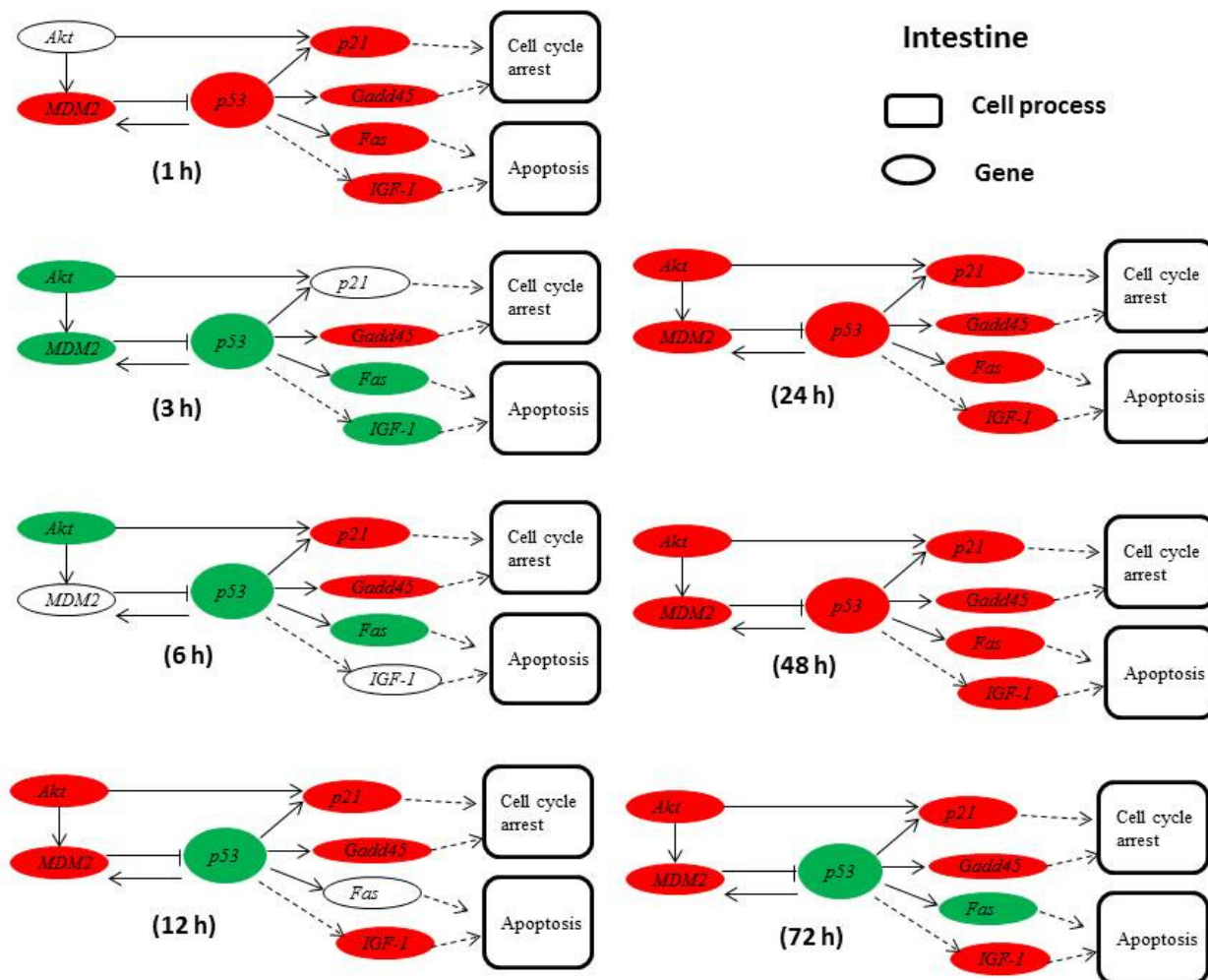
Figure 51

qPCR analysis of *p53* in the intestine of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 52

qPCR analysis of *p21* in the intestine of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
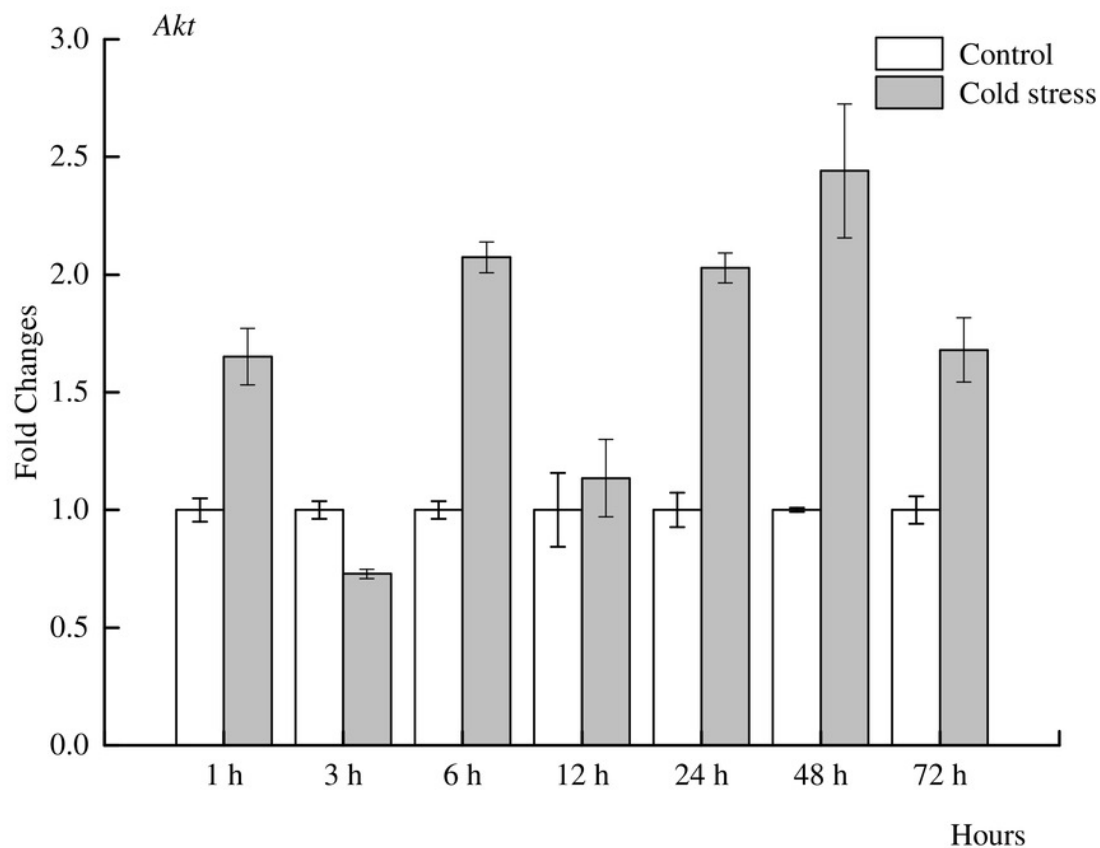
Figure 53

qPCR analysis of *MDM2* in the intestine of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 54

qPCR analysis of *IGF-1* in the intestine of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
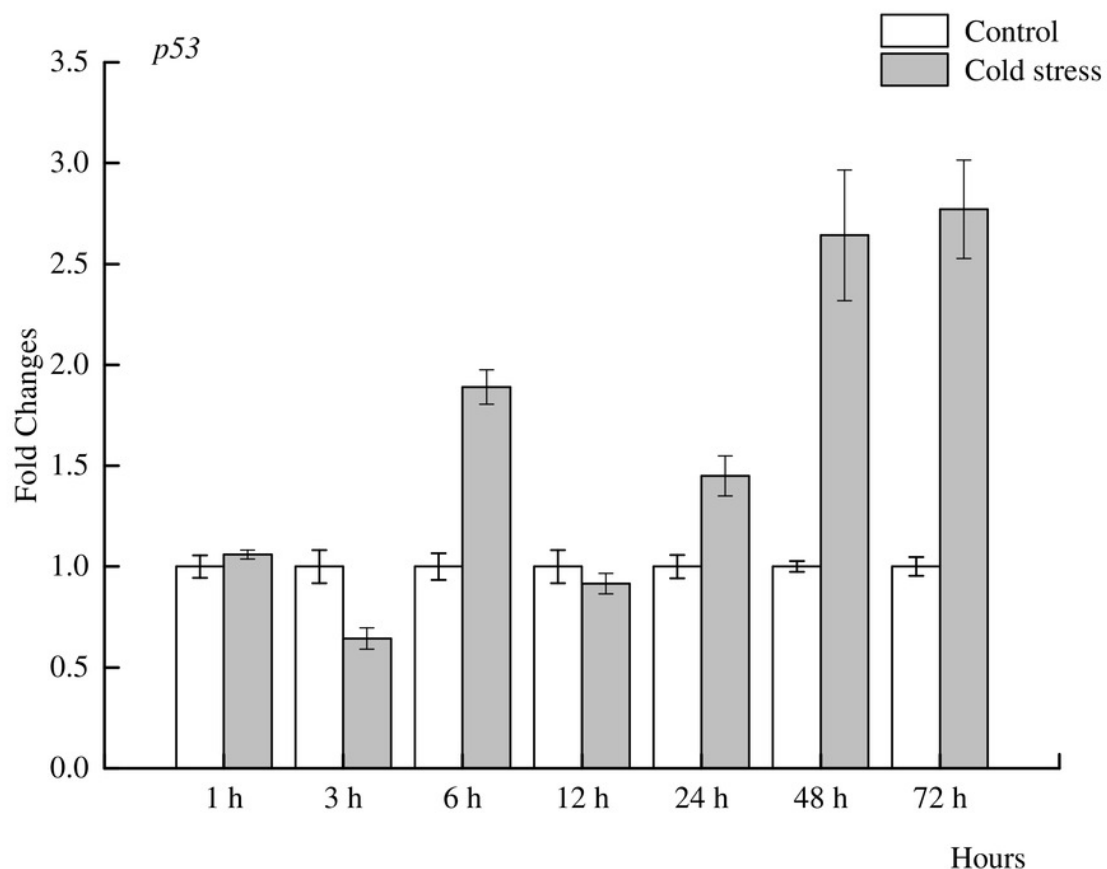
Figure 55

Gadd45

Figure 56

qPCR analysis of *Fas* in the intestine of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 57

putative gene networks based on qPCR datas in intestine

Figure 58

qPCR analysis of *Akt* in the heart of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 59

qPCR analysis of *p53* in the heart of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
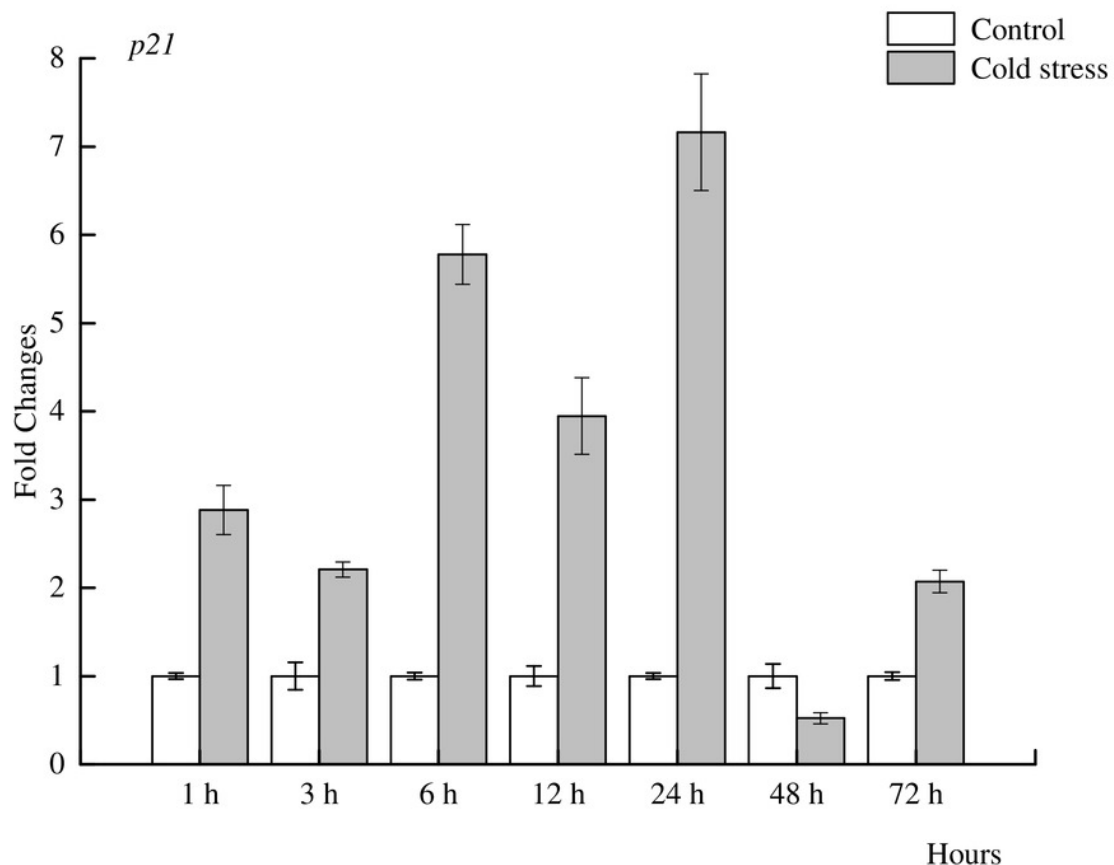
Figure 60

qPCR analysis of *p21* in the heart of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)

Figure 61

qPCR analysis of *MDM2* in the heart of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
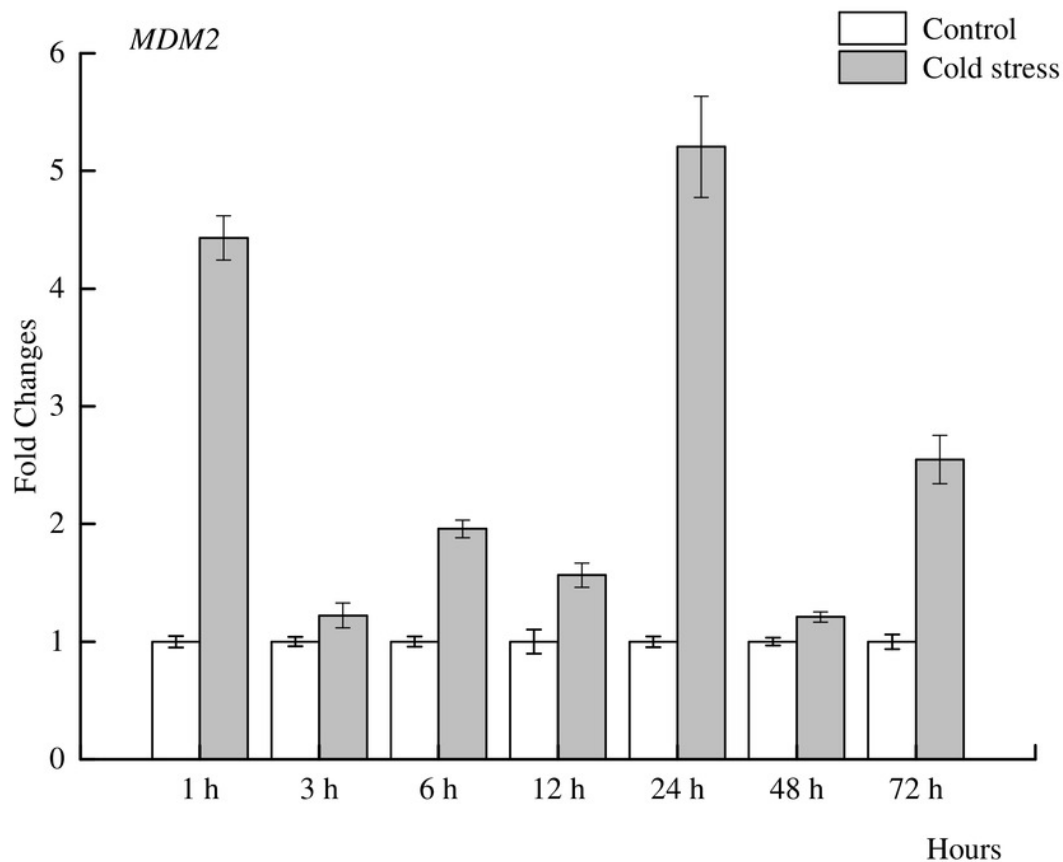
Figure 62

qPCR analysis of *IGF-1* in the heart of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
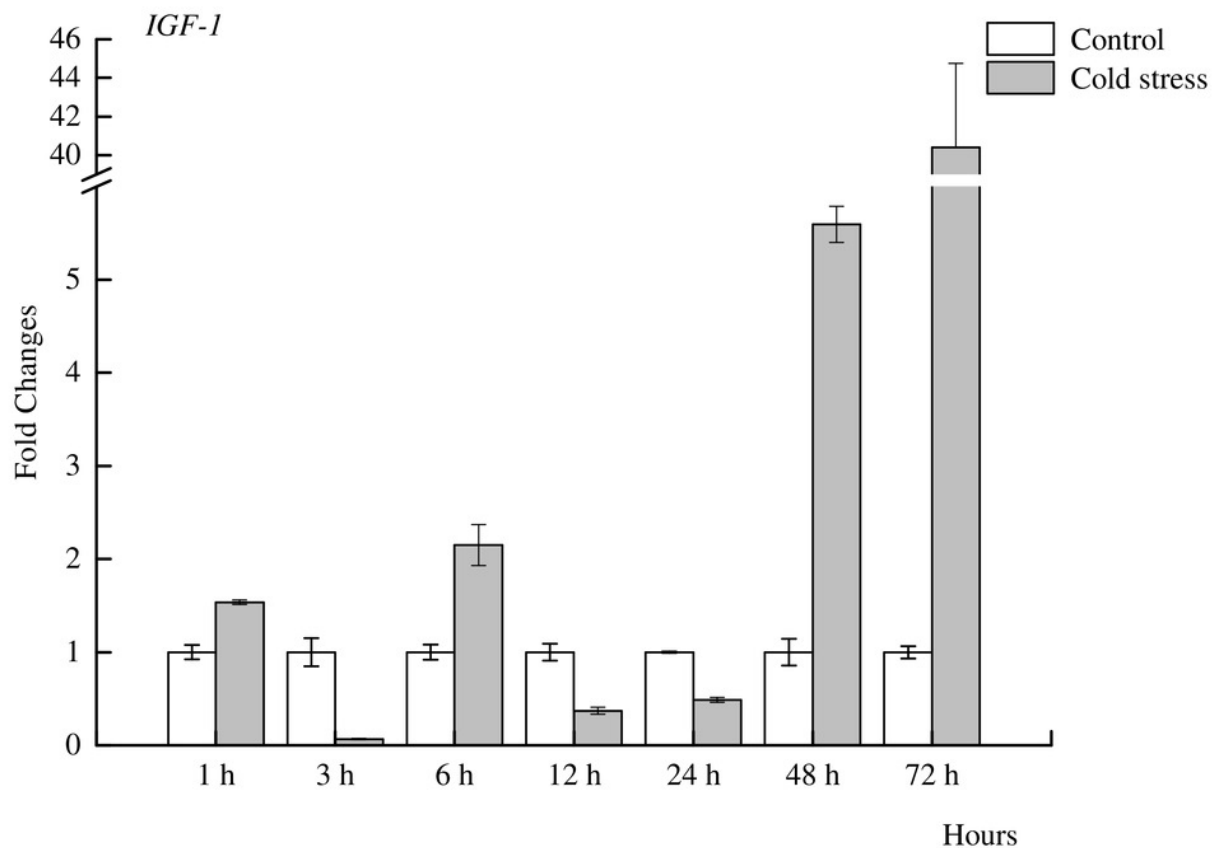
Figure 63

qPCR analysis of Gadd45 in the heart of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
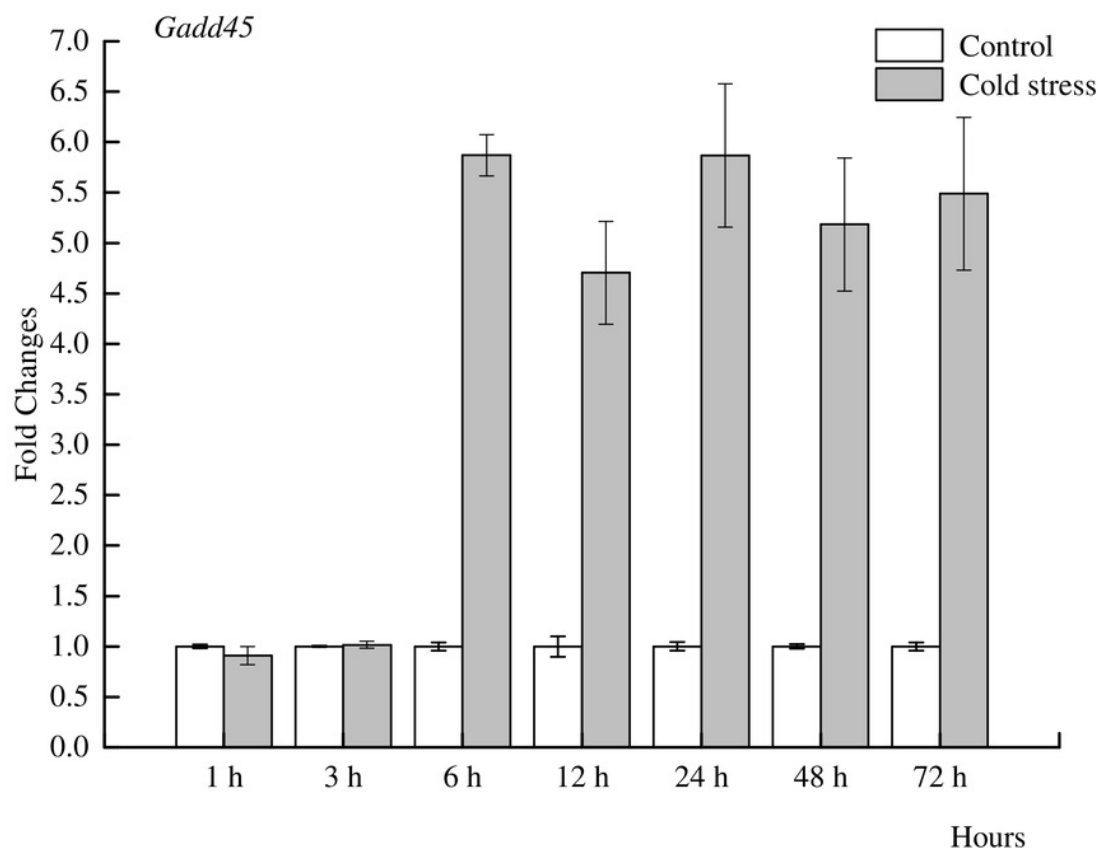
Figure 64

qPCR analysis of *Fas* in the heart of large yellow croaker under acute cold stress at different time point (1, 3, 6, 12, 24, 72 h)
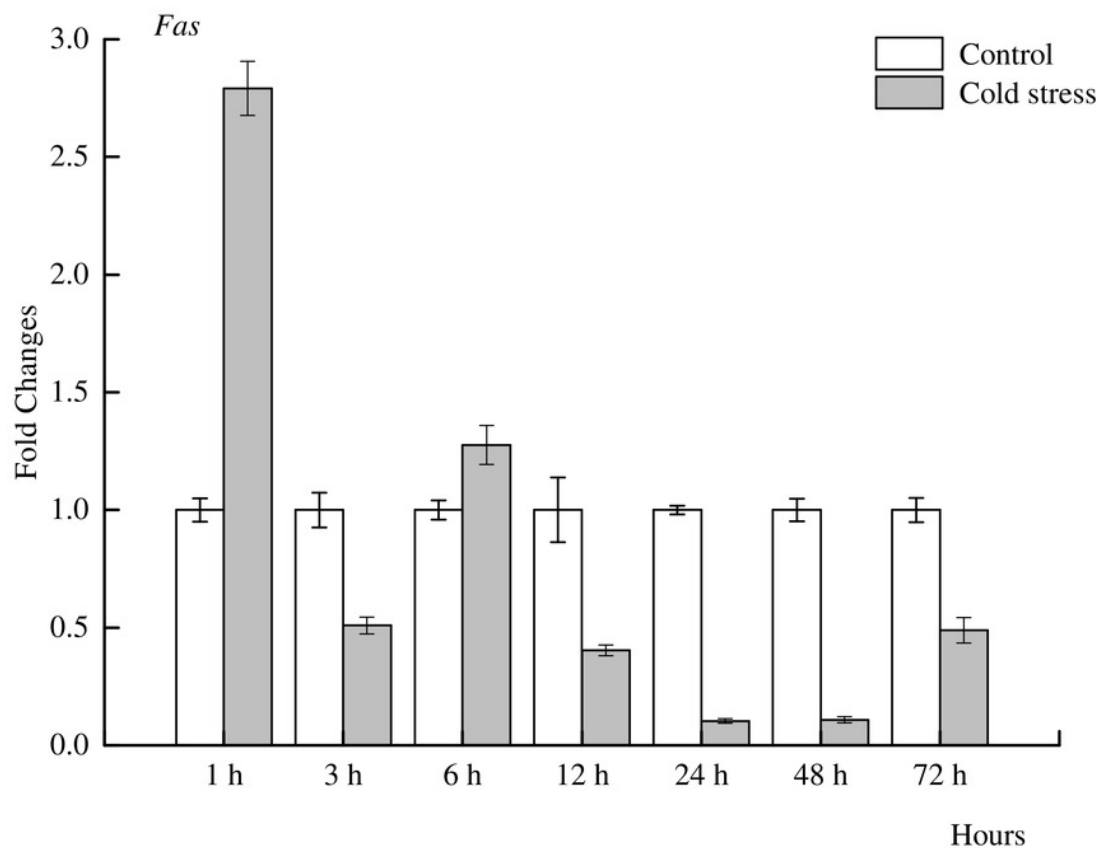
Figure 65

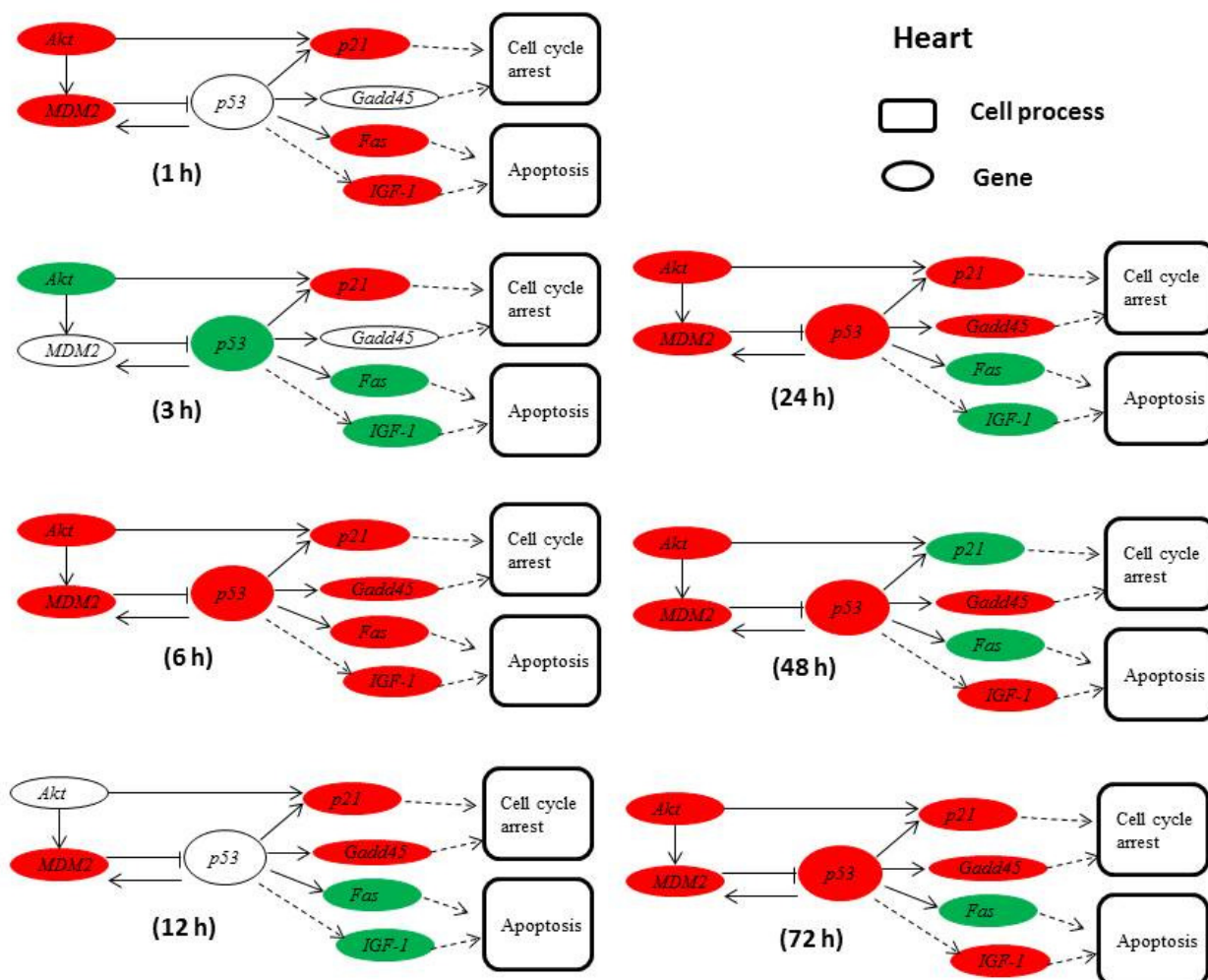putative gene networks based on qPCR datas in heart

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Table 1(on next page)

Primers for quantitative real time PCR

1   **Table 1**   Primers for quantitative real time PCR.

Gene	Primer sequence (5'—3')	Gene	Primer sequence (5'—3')
β-actin	F: TCGGTATGGAATCTTGCG	*Fas*	F: CACTCCAGCAGGGAAATGGA
	R: GTATTTACGCTCAGGTGGG		R: GCCATTTTGCTACGTCTCGC
P53	F: ACTACTGCCGGCCTAATGTG	*Akt*	F: TGCCCCAGCATGAATGAAGT
	R: GCAAACTGCATGGTTGGAGG		R: GTTGTGGTCACTGGACACCT
MDM2	F: TAGACGCCGTGCATGGATTT	*Gadd45*	F: ATCAACGTGGTGCGAGTCAA
	R: CCAGTTTGTTGTCATCGGCG		R: CATTGCAGTAGCGTGTGCAG
P21	F: GGGAAATGGCACCAATGTCG	*IGF-1*	F: GTTCATTTTCGCCGGGCTTT
	R: GACGAAGAAGATGTCCGCCT		R: ACAGCACATCGCACTCTTGA

2